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THE
BIOLOGICAL BULLETIN

PUBLISHED BY
THE MARINE BIOLOGICAL LABORATORY

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THE BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

THE MARINE BIOLOGICAL LABORATORY

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Charles Packard, Woods Hole, Massachusetts
A. C. Redfield, Woods Hole Oceanographic Institution
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Alfred H. Sturtevant, California Institute of Technology
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Teru Hayashi, Columbia University
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Sears Crowell, Indiana University
Albert I. Lansing, University of Pittsburgh Medical School
William D. McElroy, Johns Hopkins University
C. Ladd Prosser, University of Illinois
S. Meryl Rose, Wesleyan University
Mary Sears, Woods Hole Oceanographic Institution
Albert Tyler, California Institute of Technology

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II. ACT OF INCORPORATION

No. 3170

COMMONWEALTH OF MASSACHUSETTS

Be It Known, That whereas Alpheus Hyatt, William Sanford Stevens, William T. Sedgwick, Edward G. Gardiner, Susan Minns, Charles Sedgwick Minot, Samuel Wells, William G. Farlow, Anna D. Phillips, and B. H. Van Vleck have associated themselves with the intention of forming a Corporation under the name of the Marine Biological Laboratory, for the purpose of establishing and maintaining a laboratory or station for scientific study and investigation, and a school for instruction in biology and natural history, and have complied with the provisions of the statutes of this Commonwealth in such case made and provided, as appears from the certificate of the President, Treasurer, and Trustees of said Corporation, duly approved by the Commissioner of Corporations, and recorded in this office;

Now, therefore, I, HENRY B. PIERCE, Secretary of the Commonwealth of Massachusetts, *do hereby certify* that said A. Hyatt, W. S. Stevens, W. T. Sedgwick, E. G. Gardiner, S. Minns, C. S. Minot, S. Wells, W. G. Farlow, A. D. Phillips, and B. H. Van Vleck, their associates and successors, are legally organized and established as, and are hereby made, an existing Corporation, under the name of the MARINE BIOLOGICAL LABORATORY, with the powers, rights, and privileges, and subject to the limitations, duties, and restrictions, which by law appertain thereto.

Witness my official signature hereunto subscribed, and the seal of the Commonwealth of Massachusetts hereunto affixed, this twentieth day of March, in the year of our Lord One Thousand Eight Hundred and Eighty-Eight.

[SEAL]

HENRY B. PIERCE,
Secretary of the Commonwealth.

III. BY-LAWS OF THE CORPORATION OF THE MARINE BIOLOGICAL LABORATORY

I. The members of the Corporation shall consist of persons elected by the Board of Trustees.

II. The officers of the Corporation shall consist of a President, Vice President, Director, Treasurer, and Clerk.

III. The Annual Meeting of the members shall be held on the Friday following the second Tuesday in August in each year at the Laboratory in Woods Hole, Massachusetts, at 9:30 A.M., and at such meeting the members shall choose by ballot a Treasurer and a Clerk to serve one year, and eight Trustees to serve four years, and shall transact such other business as may properly come before the meeting. Special meetings of the members may be called by the Trustees to be held at such time and place as may be designated.

IV. Twenty-five members shall constitute a quorum at any meeting.

V. Any member in good standing may vote at any meeting, either in person or by proxy duly executed.

VI. Inasmuch as the time and place of the Annual Meeting of members are fixed by these By-laws, no notice of the Annual Meeting need be given. Notice of any special meeting of members, however, shall be given by the Clerk by mailing notice of the time and place and purpose of such meeting, at least fifteen (15) days before such meeting, to each member at his or her address as shown on the records of the Corporation.

VII. The Annual Meeting of the Trustees shall be held promptly after the Annual Meeting of the Corporation at the Laboratory in Woods Hole, Mass. Special meetings of the Trustees shall be called by the President, or by any seven Trustees, to be held at such time and place as may be designated, and the Secretary shall give notice thereof by written or printed notice, mailed to each Trustee at his address as shown on the records of the Corporation, at least one (1) week before the meeting. At such special meeting only matters stated in the notice shall be considered. Seven Trustees of those eligible to vote shall constitute a quorum for the transaction of business at any meeting.

VIII. There shall be three groups of Trustees:

(A) Thirty-two Trustees chosen by the Corporation, divided into four classes, each to serve four years. After having served two consecutive terms of four years each, Trustees are ineligible for re-election until a year has elapsed. In addition, there shall be two groups of Trustees as follows:

(B) Trustees ex officio, who shall be the President and Vice President of the Corporation, the Director of the Laboratory, the Associate Director, the Treasurer, and the Clerk:

(C) Trustees Emeriti, who shall be elected from *present* or *former* Trustees by the Corporation. Any regular Trustee who has attained the age of seventy years shall continue to serve as Trustee until the next Annual Meeting of the Corporation, whereupon his office as regular Trustee shall become vacant and be filled by election by the Corporation and he shall become eligible for election as Trustee *Emeritus* for life. The Trustees *ex officio* and *Emeriti* shall have all the rights of the Trustees except that Trustees *Emeritus* shall not have the right to vote.

The Trustees and officers shall hold their respective offices until their successors are chosen and have qualified in their stead.

IX. The Trustees shall have the control and management of the affairs of the Corporation; they shall elect a President of the Corporation who shall also be Chairman of the Board of Trustees and who shall be elected for a term of five years and shall serve until his successor is selected and qualified; and shall also elect a Vice President of the Corporation who shall also be the Vice Chairman of the Board of Trustees and who shall be elected for a term of five years and shall serve until his successor is selected and qualified; they shall appoint a Director of the Laboratory; and they may choose such other officers and agents as they may think best; they may fix the compensation and define the duties of all the officers and agents; and may remove them, or any of them, except those chosen by the members, at any time; they may fill vacancies occurring in any manner in their own number or in any of the offices. The Board of Trustees shall have the power to choose an Executive Committee from their own number, and to delegate to such Committee such of their own powers as they may deem expedient. They shall from time to time elect members to the Corporation upon such terms and conditions as they may think best.

X. The Associates of the Marine Biological Laboratory shall be an unincorporated group of persons (including associations and corporations) interested in the Laboratory and shall be organized and operated under the general supervision and authority of the Trustees.

XI. The consent of every Trustee shall be necessary to dissolution of the Marine Biological Laboratory. In case of dissolution, the property shall be disposed of in such manner and upon such terms as shall be determined by the affirmative vote of two-thirds of the Board of Trustees.

XII. The account of the Treasurer shall be audited annually by a certified public accountant.

XIII. These By-laws may be altered at any meeting of the Trustees, provided that the notice of such meeting shall state that an alteration of the By-laws will be acted upon.

IV. REPORT OF THE DIRECTOR

To: The Trustees of the Marine Biological Laboratory
Gentlemen:

I submit herewith the report of the seventy-fourth session of the Marine Biological Laboratory.

1. Plant Additions

During the past winter six cottages were built on the Devil's Lane Tract with funds which were generously granted the Laboratory by the James Foundation of New York. These are winterized cottages which can be available throughout the year to visiting scientists at the Laboratory. This significant addition to the Laboratory's housing will help to ease a difficult situation.

2. Systematics–Ecology Program

Dr. Melbourne R. Carriker has been appointed Director of the Systematics-Ecology Program, the appointment to become effective on September 1, 1962. Dr. Carriker took his Bachelor of Science degree at Rutgers in 1939 and his Doctor of Philosophy degree at the University of Wisconsin in 1943. He has been a member of the zoology staffs at Rutgers University and at the University of North Carolina and has served as Supervisory Fishery Research Biologist at the Oxford, Maryland, Biological Laboratory of the Bureau of Commercial Fisheries. The program under Dr. Carriker's direction will be financed in part by a grant from the Ford Foundation.

3. Personnel Changes

This past summer Dr. Eugene P. Odum completed his five-year term as head of the training program in Marine Ecology and is being succeeded by Dr. John H. Ryther. Dr. J. Woodland Hastings takes charge of the training program in Physiology, succeeding Dr. W. D. McElroy. Dr. C. B. Metz will head up the newly established training program in Fertility Problems and Dr. James D. Ebert will take over the direction of the Embryology training program. The Laboratory has been most fortunate in the men it has enlisted as heads of its various training programs.

4. Naming of Buildings

At its midwinter meeting the Board of Trustees named its two main laboratories in honor of two former Directors of the Laboratory. The main laboratory will be known as the Lillie Building in honor of Frank R. Lillie, Director from 1908 to 1926 and President of the Corporation from 1925 to 1942. Dr. Lillie is in a large measure responsible for the modern development of the Laboratory. The new laboratory building will be the Whitman Building in honor of Charles O. Whitman, Director from 1888 to 1908, who guided the destinies of the Laboratory through a very critical period of its development. Suitable plaques will be placed on these buildings recording this action of the Board of Trustees.

5. Grants, Contracts and Contributions in Support of Laboratory Activities, including Training Grants

The total income from these services of support amounted to $302,716 in 1961. This represents 31% of the $988,172 total income and is made up of support from the following:

Training grants from NIH and NSF, support for regular research activities from NIH, NSF, AEC and ONR and gifts from the MBL Associates, Josephine C. Crane Foundation, The Rockefeller Foundation, and the following pharmaceutical companies: The Merck Co. Foundation, C.I.B.A. Pharmaceutical Products, Inc., Abbott Laboratories, Schering Foundation Inc., Eli Lilly and Company, The Upjohn Company, Wallace Laboratories and the Olin Mathieson Chemical Corporation Charitable Trust.

6. Deaths

During the course of the year the Laboratory lost two of its very eminent members through death, Dr. Otto Loewi and Dr. G. Failla. Both of these scientists conferred distinction on the Laboratory through their membership in the Corporation and their scientific activities through many summers of research activity at the Laboratory.

Also we must note the passing of Mr. Alton J. Pierce of the technical staff who was highly regarded for his kind good nature, cooperativeness and technical skill.

Respectfully submitted,
PHILIP B. ARMSTRONG
Director

ZOOLOGY

I. CONSULTANTS

F. A. BROWN, JR., Professor of Zoology, Northwestern University
LIBBIE H. HYMAN, American Museum of Natural History
ALFRED C. REDFIELD, Woods Hole Oceanographic Institution

II. Instructors

Clark P. Read, Professor of Biology, Rice University, in charge of the course
Bernard L. Strehler, Chief, Cellular and Comparative Physiology, Division of Gerontology, National Institutes of Health
Richard C. Sanborn, Professor of Zoology, Purdue University
James Case, Associate Professor of Zoology, State University of Iowa
Earl Segal, Assistant Professor of Biology, Rice University
Charles E. Jenner, Professor of Zoology, University of North Carolina
W. D. Russell Hunter, Department of Zoology, University of Glasgow, Scotland, U. K.

III. Assistants

David C. Grant, Yale University
Stephen Smith, Wesleyan University

EMBRYOLOGY

I. Instructors

Nelson T. Spratt, Jr., Professor of Zoology, University of Minnesota, in charge of the course
Philip Grant, Assistant Professor of Pathobiology, The Johns Hopkins University
John W. Saunders, Jr., Professor of Zoology, Marquette University
Tore Hultin, Wenner-Grens Institute, Stockholm, Sweden
Aaron Moscona, Professor of Zoology, University of Chicago
Laurens Ruben, Assistant Professor of Biology, Reed College

II. Laboratory Assistants

John Arnold, Oberlin College
Richard Whittaker, Yale University

PHYSIOLOGY

I. Consultants

Merkel H. Jacobs, Professor of Physiology, University of Pennsylvania
Otto Loewi, Professor of Pharmacology, New York University School of Medicine
Arthur K. Parpart, Professor of Biology, Princeton University
Albert Szent-Györgyi, Director, Institute for Muscle Research, Marine Biological Laboratory

II. Instructors

W. D. McElroy, Director, McCollum-Pratt Institute, Johns Hopkins University; in charge of the course
Philip E. Hartman, Associate Professor of Biology, Johns Hopkins University
Timothy H. Goldsmith, Yale University
Howard K. Schachman, Virus Laboratory, University of California, Berkeley
Robert B. Loftfield, Massachusetts General Hospital
André Jagendorf, McCollum-Pratt Institute, Johns Hopkins University
J. Woodland Hastings, Assistant Professor of Biochemistry, University of Illinois

III. Laboratory Assistant

Luis Otero, University of Puerto Rico, Rio Piedras

BOTANY

I. Consultant

William Randolph Taylor, Professor of Botany, University of Michigan

II. Instructors

Richard C. Starr, Professor of Botany, Indiana University, in charge of the course
Walter R. Herndon, Associate Professor of Botany, University of Alabama
John M. Kingsbury, Associate Professor of Botany, Cornell University
Tyge Christiensen, Institut for Sporeplanter, University of Copenhagen

III. Laboratory Assistants

Austin Brooks, Department of Biology, Wabash College
Wayne Nichols, Department of Biology, University of Alabama

ECOLOGY

I. Consultants

Paul Galtsoff, U. S. Fish and Wildlife Service, Woods Hole
Alfred C. Redfield, Woods Hole Oceanographic Institution
Bostwick H. Ketchum, Woods Hole Oceanographic Institution
Edwin T. Moul, Rutgers University
Charles E. Jenner, University of North Carolina
Howard T. Odum, University of Texas

II. Instructors

Eugene P. Odum, Alumni Foundation Professor of Zoology, University of Georgia, in charge of the course
John H. Ryther, Marine Biologist, Woods Hole Oceanographic Institution
Howard L. Sanders, Woods Hole Oceanographic Institution
Walter R. Taylor, Chesapeake Bay Institute, Johns Hopkins University

III. Laboratory Assistants

Elijah V. Swift, Johns Hopkins University
William S. Broughton, University of Georgia

1. The Laboratory Staff

Mrs. Deborah Lawrence Harlow, Librarian
Carl O. Schweidenback, Manager, Supply Department
Irvine L. Broadbent, Office Manager
Robert Kahler, Superintendent, Buildings and Grounds
Robert B. Mills, Manager, Department of Research Service

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Mrs. Vivien R. Brown
Mrs. Virginia M. Morehouse
Mrs. Marion C. Chase
Mrs. Vivian I. Manson
Mrs. Ruth Mayo

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Noreen Richmond
Doris Ricker
Teena B. Chase
Albert K. Neal

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Eldon P. Allen
Francis Cavanaugh
Manuel P. Dutra
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Gardner F. Gayton
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Russell F. Lewis
Alan G. Lunn
Ellen T. Nickelson
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Caroline McDaniel
Seaver R. Harlow
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Arnold Bowden
Robert O. Lehy
Robert M. Perry
Mrs. Patricia Tavares
Bruno F. Trapasso
John J. Valois
Jared L. Vincent
Hallett S. Wagstaff

2. Investigators; Lalor and Lillie Fellows; and Students

Independent Investigators, 1961

Adams, Ralph G., Physicist, National Institutes of Health
Adelberg, Edward A., Professor of Bacteriology, University of California
Adelman, William J., Jr., Physiologist, National Institutes of Health
Aljure, Emilio, Universidad del Valle, Cali, Colombia
Allen, M. Jean, Professor of Biology, Wilson College
Armstrong, Philip B., Professor of Anatomy, State University of New York College of Medicine, at Syracuse
Atwood, Kimball C., Professor of Microbiology, University of Illinois
Austin, C. R., Member of Research Staff, National Institute for Medical Research, London
Baird, Spencer L., Associate, Institute for Muscle Research
Barth, L. G., Professor of Zoology, Columbia University
Baylor, Martha B., Marine Biological Laboratory
Bennett, Michael V. L., Assistant Professor of Neurology, College of Physicians and Surgeons
Bernstein, Maurice H., Assistant Professor of Anatomy, Wayne State University
Beukers, Robert, Staff Member, Technological University, Delft, Netherlands

Binstock, Leonard, Electronic Engineer, National Institutes of Health
Blake, John M., Chief Biologist, Aquacultural Research Corporation
Blum, John L., Professor of Biology, Canisius College
Bosler, Robert, Dept. of Pharmacology, Harvard Medical School
Brown, Frank A., Jr., Morrison Professor of Biology, Northwestern University
Bryant, S. H., Assistant Professor of Pharmacology, University of Cincinnati, College of Medicine
Burke, Joseph A., Assistant Professor of Biology, Loyola College
Campbell, James W., Instructor in Biology, Rice University
Carlson, Francis D., Professor of Biophysics, Johns Hopkins University
Case, James, Associate Professor of Zoology, University of Iowa
Chaet, Alfred B., Associate Professor of Biology, American University
Chandler, William K., Medical Officer, National Institutes of Health
Cheney, Ralph Holt, Professor of Biology, Brooklyn College
Child, Frank M., Assistant Professor of Zoology, University of Chicago
Christensen, A. Kent, Instructor in Anatomy, Harvard Medical School
Christensen, Tyge, University of Copenhagen, Institute of Thallophyta
Claff, C. Lloyd, Research Associate in Surgery, Harvard Medical School
Clark, Arnold M., Professor of Biological Sciences, University of Delaware
Cole, Kenneth S., Chief, Laboratory of Biophysics, National Institutes of Health
Collier, Jack R., Marine Biological Laboratory
Colwin, Arthur L., Professor of Biology, Queens College
Colwin, Laura Hunter, Queens College
Cooperstein, Sherwin J., Associate Professor of Anatomy, Western Reserve University
Copeland, Eugene, Professor of Zoology, Tulane University
Costello, Donald Paul, Kenan Professor of Zoology, University of North Carolina
Crane, Robert K., Associate Professor of Biological Chemistry, Washington University Medical School
Crowell, Sears, Associate Professor of Zoology, Indiana University
Csapo, Arpad I., Associate Professor, Rockefeller Institute
Dalton, John C., Assistant Professor of Biology, University of Buffalo
Dettbarn, Wolf-Dietrich, Assistant Professor of Neurology, College of Physicians and Surgeons
Diamond, Jack, Harvard Medical School
Edds, Mac V., Jr., Professor of Biology, Brown University
Failla, G., Senior Physicist Emeritus, Argonne National Laboratory
Ferguson, James J., Jr., Assistant Professor, University of Pennsylvania
Fischer, Siegmund, Associate in Research, Albert Einstein College of Medicine
Fitzpatrick, Thomas B., Professor of Dermatology, Massachusetts General Hospital
Frazier, Howard S., Massachusetts General Hospital
Furshpan, Edwin J., Associate in Neurophysiology, Harvard Medical School
Gainer, Harold, Research Associate, College of Physicians and Surgeons
Gilman, Lauren C., Associate Professor of Zoology, University of Miami
Girardier, Lucien, Research Associate, College of Physicians and Surgeons
Glade, Richard W., Assistant Professor of Zoology, University of Vermont
Goldring, Irene P., Assistant Professor of Research Surgery, Albert Einstein College of Medicine
Goldsmith, Timothy H., Yale University
Grant, Philip, Assistant Professor of Pathobiology, Johns Hopkins University School of Hygiene
Greif, Roger L., Associate Professor of Physiology, Cornell University Medical College
Grosch, Daniel S., Professor of Genetics, North Carolina State College
Gross, Paul R., Associate Professor of Biology, New York University
Grundfest, Harry, Professor of Neurology, College of Physicians and Surgeons
Guttman, Rita, Associate Professor of Biology, Brooklyn College
Gwilliam, Gilbert F., Assistant Professor of Biology, Reed College
Hagins, William A., Physiologist, National Institutes of Health
Hagiwara, Susumu, Professor of Zoology, University of California

Harding, Clifford V., Assistant Professor of Physiology, College of Physicians and Surgeons
Hartman, Philip E., Associate Professor of Biology, Johns Hopkins University
Harvey, Ethel Browne, Marine Biological Laboratory
Hastings, J. Woodland, Assistant Professor of Biochemistry, University of Illinois
Hathaway, Ralph R., Oceanographic Institute, Florida State University
Hayashi, Teru, Professor of Zoology, Columbia University
Hegyeli, Andrew, Institute for Muscle Research, Marine Biological Laboratory
Henley, Catherine, Research Associate, University of North Carolina
Herndon, Walter R., Associate Professor of Biology, University of Alabama
Hervey, John P., Senior Electronic Engineer, Rockefeller Institute
Herzog, Walter, Post Doctoral Fellow in Pharmacology, University of Cincinnati College of Medicine
Higman, Henry B., College of Physicians and Surgeons
Hirshfield, Henry I., Associate Professor of Biology, Washington Square College
Hoskin, Francis C. G., Assistant Professor of Neurology, College of Physicians and Surgeons
Hultin, Tore, Wenner-Grens Institute, University of Stockholm
Hunter, W. D. Russell, Lecturer in Zoology, University of Glasgow, Scotland
Hurwitz, Jerard, Associate Professor of Microbiology, New York University School of Medicine.
Isenberg, Irvin, Institute for Muscle Research, Marine Biological Laboratory
Jagendorf, André, Associate Professor, Johns Hopkins University
Janiszewski, Leszek, Master of Biology, N. Copernicus University, Poland
Jenner, Charles E., Professor of Zoology, University of North Carolina
Kaminer, Benjamin, Institute for Muscle Research, Marine Biological Laboratory
Kane, Robert E., Assistant Professor of Cytology, Dartmouth Medical School
Katz, George M., Research Associate, Columbia University
Kempton, Rudolf T., Professor of Zoology, Vassar College
Keosian, John, Professor of Biology, Rutgers, The State University
Kingsbury, John M., Associate Professor of Botany, Cornell University
Kishimoto, Uichiro, National Institutes of Health
Kleinholz, Lewis H., Professor of Biology, Reed College
Krane, Stephen M., Associate in Medicine, Harvard Medical School
Kuffler, Stephen W., Professor of Neurophysiology, Harvard Medical School
Landau, Joseph V., Chief, Oncology Section, VA Hospital, Albany, New York
Lansing, Albert I., Professor of Anatomy, University of Pittsburgh School of Medicine
Lazarow, Arnold, Professor of Anatomy, University of Minnesota
Leaf, Alexander, Associate Professor of Medicine, Harvard Medical School
Lerman, Sidney, Assistant Professor of Ophthalmology, University of Rochester
Levy, Milton, Professor of Biochemistry, New York University College of Dentistry
Lochhead, John H., Professor of Zoology, University of Vermont
Loewenstein, Werner R., Associate Professor of Physiology, College of Physicians and Surgeons
Loftfield, Robert B., Associate Biochemist, Massachusetts General Hospital
London, Irving M., Professor of Medicine, Albert Einstein College of Medicine
Lorand, L., Associate Professor of Chemistry, Northwestern University
de Lorenzo, A. J., Director Anatomical and Pathological Laboratories, Johns Hopkins University
Love, Warner E., Assistant Professor of Biophysics, Johns Hopkins University
Mahler, H. R., Professor of Chemistry, Indiana University
Marsland, Douglas, Professor of Biology, Washington Square College
Mateyko, G. M., Assistant Professor of Biology, Washington Square College
McElroy, William D., Director, McCollum-Pratt Institute, Johns Hopkins University
Metz, Charles B., Professor of Zoology, Florida State University
Metz, Charles W., Emeritus Professor of Zoology, University of Pennsylvania
Middlebrook, William R., Institute for Muscle Research, Marine Biological Laboratory
Milkman, Roger D., Associate Professor of Zoology, Syracuse University
Miller, Faith S., Assistant Professor of Anatomy, Tulane University
Miller, James A., Professor of Anatomy, Tulane University

Moore, Richard O., Professor, Ohio State University
Morrill, John B., Assistant Professor, Wesleyan University
Moscona, A. A., Professor of Zoology, University of Chicago
Mullins, Lorin J., Professor of Biophysics, University of Maryland
Musacchia, X. J., Associate Professor of Biology, Saint Louis University
Nace, Paul Foley, Professor of Biology, McMaster University
Nagi, Toshio, Research Associate, University of Illinois
Nelson, Leonard, Associate Professor of Physiology, Emory University
Odum, Eugene P., Professor of Zoology, University of Georgia
Palincsar, Edward E., Assistant Professor of Biology, Loyola University
Parker, Johnson, Assistant Professor of Plant Physiology, Yale University
Parpart, Arthur K., Professor of Biology, Princeton University
Penman, Sheldon, Assistant Professor of Physics, College of Physicians and Surgeons
Person, Philip, Chief, VA Hospital, Brooklyn
Petrie, Asenath, Research Associate, Harvard University and London University
Potter, David, Associate in Neurophysiology, Harvard Medical School
Prosser, C. Ladd, Professor of Physiology, University of Illinois
Rapport, Maurice M., Professor of Biochemistry, Albert Einstein College of Medicine
Read, Clark P., Professor of Biology, Rice University
Rebhun, Lionel I., Assistant Professor of Biology, Princeton University
Reuben, John P., Research Associate, College of Physicians and Surgeons
Rieser, Peter, Head, Department of Biology, St. John Fisher College
Rockstein, Morris, Associate Professor of Physiology, New York University College of Medicine
Rose, S. Meryl, Professor of Biology, Wesleyan University
Rosenberg, Evelyn K., Associate Professor, New York University
Rosenberg, Philip, Columbia University, College of Physicians and Surgeons
Rothstein, Howard, College of Physicians and Surgeons
Rowland, Lewis P., Assistant Professor of Neurology, College of Physicians and Surgeons
Ruben, Laurens, Assistant Professor of Biology, Reed College
Ruck, Philip R., Assistant Professor of Biology, Tufts University
Rugh, Roberts, Associate Professor of Radiology, College of Physicians and Surgeons
Rustad, Ronald C., Assistant Professor of Physiology, Florida State University
Sanborn, Richard C., Professor of Zoology, Purdue University
Sanders, Howard L., Woods Hole Oceanographic Institution
Saunders, John W., Professor of Biology, Marquette University
Schachman, Howard K., Professor of Biochemistry, University of California
Scharrer, Ernst, Professor of Anatomy, Albert Einstein College of Medicine
Schoffeniels, Ernest, Assistant Professor of Neurology, College of Physicians and Surgeons
Scott, Allan, Professor of Biology, Colby College
Scott, Sister Florence Marie, Professor of Biology, Seton Hill College
Scott, George T., Chairman, Department of Biology, Oberlin College
Segal, Earl, Assistant Professor of Biology, Rice University
Seliger, Howard H., Research Associate, McCollum-Pratt Institute, Johns Hopkins University
Senft, Alfred W., Marine Biological Laboratory
Sengel, Philippe, Collège de France
Simmons, John E., Rice University
Sjodin, Raymond A., Associate Professor of Biophysics, University of Maryland
Slifer, Eleanor H., Professor of Zoology, State University of Iowa
Smelser, George K., Professor of Anatomy, Columbia University
Sonnenblick, Benjamin, Professor of Biology, Rutgers, The State University
Spector, Abraham, Instructor, Howe Laboratory, Harvard Medical School
Speidel, Carl C., Professor of Anatomy, University of Virginia
Spindel, William, Associate Professor of Chemistry, Rutgers University
Spratt, Nelson T., Chairman, Department of Zoology, University of Minnesota
Spyropoulos, Constantine S., Neurophysiologist, National Institutes of Health
Starr, Richard C., Professor of Botany, Indiana University
Steffensen, Dale M., Associate Professor of Botany, University of Illinois

Steinbach, H. Burr, Professor of Zoology, University of Chicago
Steinhardt, Jacinto, Director, Operations Evaluation Group, Massachusetts Institute of Technology
Stetten, DeWitt, Jr., Associate Director, NIAMD, National Institutes of Health
Stone, William, Jr., Director, Ophthalmic Plastics Laboratory
Strehler, Bernard L., Chief, Cellular and Comparative Physiology Section, National Institutes of Health
Strittmatter, Philipp, Assistant Professor of Biochemistry, Washington University
Sussman, Maurice, Professor of Biology, Brandeis University
Szabo, George, Associate in Anatomy, Harvard Medical School
Szent-Gyorgyi, Albert, Director, Institute for Muscle Research, Marine Biological Laboratory
Szent-Gyorgyi, Andrew, Head of Muscle Section, Institute for Muscle Research, Marine Biological Laboratory
Tasaki, Ichiji, Chief, Special Senses Section, National Institutes of Health
Taylor, Robert E., Physiologist, National Institutes of Health
Taylor, William Randolph, Professor of Botany, University of Michigan
Taylor, W. Rowland, Assistant Professor of Oceanography, Johns Hopkins University
Teorell, Torsten, Professor, University of Uppsala
de Terra, Noel, Research Associate, Rockefeller Institute
Torch, Reuben, Associate Professor of Zoology, University of Vermont
Trams, Eberhard G., National Institutes of Health
Travis, David Monroe, Assistant Professor of Pharmacology, University of Florida
Troll, Walter, Associate Professor, New York University Medical Center
Tunik, Bernard D., Associate Professor, State University of New York, Long Island
Tweedell, Kenyon S., Assistant Professor of Biology, University of Notre Dame
deVillafranca, George W., Associate Professor of Zoology, Smith College
Villee, Claude A., Associate Professor of Biological Chemistry, Harvard University
Vincent, Walter S., Assistant Professor of Anatomy, Upstate Medical Center, State University of New York
Wald, George, Professor of Biology, Harvard University
Warren, Leonard, National Institutes of Health
Webb, H. Marguerite, Associate Professor of Biological Sciences, Goucher College
Whiting, Anna R., University of Pennsylvania
Wichterman, Ralph, Professor of Biology, Temple University
Wiercinski, Floyd J., Associate Professor of Biological Sciences, Drexel Institute of Technology
Wilber, Charles G., Dean of the Graduate School, Kent State University
Wilson, Walter L., Associate Professor of Physiology, University of Vermont
Wittenberg, Jonathan B., Associate Professor of Physiology, Albert Einstein College of Medicine
Wolff, Etienne, Professor au Collège de France
Wolsky, Alexander, Professor of Experimental Embryology, Fordham University
Wright, Paul A., Associate Professor of Zoology, University of New Hampshire
Wurtz, Robert, University of Michigan
Wyttenbach, Charles R., Instructor in Anatomy, University of Chicago
Zigman, Seymour, Research Associate, Ophthalmic Plastics Laboratory
Zimmerman, Arthur M., Assistant Professor, State University of New York, Downstate Medical Center
Zwilling, Edgar, Professor of Biology, Brandeis University

Lalor Fellows, 1961

Wolff, Etienne, Collège de France
Christensen, A. Kent, Harvard Medical School
Hathaway, Ralph, Florida State University
Sengel, Philippe, Collège de France
Sengel, M. Louise, Collège de France
Warren, Leonard, National Institutes of Health

Lillie Fellow, 1961

AUSTIN, C. R., National Institute for Medical Research, London

Grass Fellows, 1961

GRAFSTEIN, BERNICE, McGill University
HALL, ZACH, Emory University
HERZOG, WALTER, University of Cincinnati College of Medicine
WURTZ, ROBERT, University of Michigan

Beginning Investigators, 1961

ADYE, JAMES C., Johns Hopkins University
BAUER, G. ERIC, University of Minnesota
BEAMES, CALVIN G., JR., Rice University
BITO, LASZLO, Columbia University
BRINLEY, F. J., JR., Johns Hopkins Medical School
BROWN, JOEL E., Massachusetts Institute of Technology
CHERVIN, PAUL N., University of Vermont
CHRISTIANSEN, JANICE H., Temple University School of Medicine
DOOLITTLE, RUSSELL F., Harvard University Medical School
DUNHAM, PHILIP B., University of Chicago
ECKERT, ROGER O., Harvard University
EISENBERG, ROBERT S., Harvard College
FIELD, JAMES B., National Institutes of Health
FOX, STEPHEN S., University of Michigan
GASSELING, MARY T., Marquette University
HUMPHREYS, TOM DANIEL, University of Chicago
ISHIKO, NOBUSADA, Kumamoto University Medical School
JACKSON, JAMES A., Western Reserve University
KITAZUME, YOSHIJI, University of Kobe, Japan
LEIBERMAN, PAUL M., University of Vermont
MOORE, RICHARD O., Ohio State University
MORAN, JOSEPH F., Russell Sage College
SCHLESINGER, ROBERT M., National Institutes of Health
SCHUEL, HERBERT, Florida State University
SHEPHARD, DAVID C., University of Chicago
SLAYMAN, CLIFFORD L., Rockefeller Institute
SRINIVASAN, DOBLI, Columbia University
STONE, AUDREY L., National Institutes of Health
WHEELER, MAYNARD B., Columbia University

Research Assistants, 1961

ALEXANDER, DOUGLAS GORDON, University of North Carolina
ALLAWAY, ELIZABETH, Mount Holyoke College
ARNOLD, JOHN M., Oberlin College
ATLAS, MERYL, State University of New York, Upstate Medical Center
BARNWELL, FRANKLIN H., Northwestern University
BART, KENNETH, State University of New York, Upstate Medical Center
BENNETT, JOHN E., Purdue University
BENZINGER, ROLF H., Johns Hopkins University
BOLEYN, BRENDA J., Harvard Medical School
BOOKER, VIRGINIA TOD, Smith College
BOWERS, WILLIAM E., College of Physicians and Surgeons
BRADBURY, JACK W., Reed College
BREWER, JOHN M., Johns Hopkins University

Brooks, Austin, Wabash College
Broughton, William S., University of Georgia
Byrne, Sylvia A., Syracuse University
Carlin, Ira S., Plymouth, Mass.
Ceccarini, Costante, St. Peter's College
Cicak, Anna, Albert Einstein College of Medicine
Clark, Eloise E., Columbia University
Cohn, Dunell E., Oak Ridge, Tennessee
Corabi, Mary, Brooklyn, New York
Cornell, Kenneth, Institute for Muscle Research, Marine Biological Laboratory
Cox, Eleanor, University of Texas
Crosby, Gayle M., Brandeis University
Crowe, Priscilla Ann, Seton Hill College
de La Cruz, Armando, University of the Philippines
DeMuth, Simon H., College of Physicians and Surgeons
Dewel, William C., Wesleyan University
Dougherty, William J., Princeton University
Edwards, Jackie, University of Alabama
Eigner, Elizabeth Ann, Massachusetts General Hospital
Emmons, Louise H., North Carolina State College
Erskine, Mrs. Louise, Institute for Muscle Research, Marine Biological Laboratory
Evans, Thomas Edward, Florida State University
Feibel, Robert Marks, University of Cincinnati Medical College
Fisher, Sylvia Sue, Saint Louis University
Flathers, Ann R., University of New Hampshire
Foran, Elizabeth, Smith College
Francisco, Anne S., Agnes Scott College
Friedman, Sue, Smith College
van Gelder, Nico M., Harvard Medical School
Ginsberg, Allen, Brooklyn Veterans Administration Hospital
Ginsberg, Myron D., Wesleyan University
Grabske, Robert Jerold, University of Kansas
Grant, David C., Yale University
Grant, Robert J., Columbia University
Griggs, Robert C., University of Delaware
Grimes, Marian, Western Reserve University
Gurry, Susan M., Smith College
Gutknecht, John, University of North Carolina
Habas, Linda B., University of Illinois
Hall, Prudence J., Princeton University
Hardy, Donna Joy, Johns Hopkins University
Hayward, George Edward, Washington University
Heck, Henry d'Arcy, Princeton University
Hinshaw, Carolyn Jo, Indiana University
Hirsch, Regina, New York State University, Downstate Medical Center
Hufnagel, Linda A., University of Vermont
Jackson, Thomas John, Jefferson Medical College
Karstadt, Myron, Queens College
Kayser, Eleonore, McCollum-Pratt Institute, Johns Hopkins University
Kent, Barbara, New York City
Kielich, J. C., Massachusetts Eye and Ear Infirmary
Kimball, Frances, Reed College
Kitzman, William B., Rice University
Kusano, Kiyoshi, Tokyo Medical-Dental University
Lamborg, Marvin R., Hunting Laboratories, Massachusetts General Hospital
Lang, Norma J., Indiana University
Laufenberg, Henry J., Jr., Hahnemann Medical College
Lee, Thomas W., Rice University

Leitnor, Leo G., National Institutes of Health
Lenox, Marilyn, Muskingum College
Levin, Stephen, Wesleyan University
Lieberman, Irving, University of Pittsburgh School of Medicine
Lillibridge, Jacqueline S., State University of New York, Downstate Medical Center
Lindall, Arnold, Jr., University of Minnesota
Malinou, Sheldon H., New York University College of Dentistry
Marburg, Kenneth, Johns Hopkins University
Maren, Peter H., University of Florida College of Medicine
McCarthy, Joan, Colby College
McDonald, Kay, Rice University
McLaughlin, Jane A., Institute for Muscle Research, Marine Biological Laboratory
Miller, Richard L., University of Chicago
Morrissey, James D., Upstate Medical Center, State University of New York
Naumann, Christine, Smith College
Newton, Sandra, Rice University
Nichols, Herbert W., University of Alabama
Novak, Robert L., University of Delaware
Nystrom, Richard A., University of Illinois
Olivo, Richard F., Columbia University
Orlando, Joseph C., Loyola College
Otero-Vilardebo, Luis R., University of Puerto Rico
Pax, Ralph A., Purdue University
Perry, Barbara, Institute for Muscle Research, Marine Biological Laboratory
Philpott, Charles W., Tulane University
Philpott, Loralee L., Tulane University
Philpott, Delbert, Institute for Muscle Research, Marine Biological Laboratory
Podleski, Thomas, College of Physicians and Surgeons
Powers, Joseph A., Wesleyan University
Purple, Richard L., Rockefeller Institute
Randall, Virginia D., Jackson College
Ray, Frances, New York University
Rodgers, Patricia, State University of New York, Downstate Medical Center
Rose, Jeannette, Vassar College
Rose, Robert, American University
Rosenbluth, Raja, Columbia University
Runk, Ruth C., Institute for Muscle Research, Marine Biological Laboratory
Sandlin, Ronald A., National Institutes of Health
Schroeder, Thomas E., Northwestern University
Schur, Matthew A., Oberlin College
Scott, Nancy J., University of Vermont
Sheppard, David Elson, Johns Hopkins University
Sheridan, William Francis, University of Florida
Smith, Marie F., Johns Hopkins University
Smith, Stephen D., Wesleyan University
Spencer, Joyce, Harvard Medical School
Spiegel, Jeanette, National Institutes of Health
Stahl, Mrs. Ruth C., Johns Hopkins University
Stevens, Jean M., Reed College
Sweney, Laura A., University of Minnesota
Swift, Elijah, Johns Hopkins University—Chesapeake Bay Institute
Szent-Gyorgyi, Eve, Institute for Muscle Research, Marine Biological Laboratory
Szent-Gyorgyi, Gyula, Institute for Muscle Research, Marine Biological Laboratory
Szent-Gyorgyi, Marta, Institute for Muscle Research, Marine Biological Laboratory
Watkins, Dudley T., Western Reserve Medical School
Watters, Christopher, University of Notre Dame
Weinstock, Michael S., American University
Weis, Peddrick, New York University College of Dentistry

Westhoff, D. Douglas, Saint Louis University
Whittaker, J. Richard, Yale University
Williams, Georgia J., Wilson College
Wood, Barry F., Syracuse University
Yanosik, Harold Jon, National Institutes of Health

Library Readers, 1961

Adams, Elijah, Professor of Pharmacology, Saint Louis University School of Medicine
Ball, Eric G., Professor of Biological Chemistry, Harvard Medical School
Bodansky, Oscar, Chief, Sloan-Kettering Institute for Cancer Research
Buck, John, Physiologist, National Institutes of Health
Burbanck, W. D., Professor of Biology, Emory University
Butler, Elmer G., Osborn Professor of Biology, Princeton University
Chase, Aurin M., Associate Professor of Biology, Princeton University
Clement, A. C., Professor of Biology, Emory University
Clifford, Sister Adele, Professor of Biology, College of Mount St. Joseph
Cohen, Seymour S., Professor of Biochemistry, University of Pennsylvania
Davis, Bernard D., Professor of Bacteriology, Harvard Medical School
Eisen, Herman N., Professor of Microbiology, Washington University
Flesch, Peter, Associate Professor of Research Dermatology, University of Pennsylvania
Fries, E. F. B., Professor, City College of New York
Gabriel, Mordecai L., Associate Professor of Biology, Brooklyn College
Ginsberg, Harold S., Chairman, Department of Microbiology, University of Pennsylvania
Gurewich, Vladimir, Associate Physician, Cornell Division, Bellevue Hospital
Hoberman, Henry D., Professor of Biochemistry, Albert Einstein College of Medicine
Hotchin, John E., Assistant Director, New York State Department of Health
Hurwitz, Charles, VA Hospital, Albany
Isselbacher, Kurt J., Assistant Professor of Medicine, Harvard Medical School
Jacobs, M. H., Emeritus Professor of General Physiology, University of Pennsylvania
Kabot, Elvin A., Professor of Microbiology, College of Physicians and Surgeons
Karush, Fred, Professor of Microbiology, University of Pennsylvania School of Medicine
Klein, Morton, Professor of Microbiology, Temple University
Klotz, Irving M., Professor of Chemistry, Northwestern University
Knobil, Ernst, Assistant Professor of Physiology, Harvard University
Levine, Rachmiel, Professor of Medicine, New York Medical College
Lineaweaver, Thomas H., Woods Hole, Massachusetts
Lowenfeld, Irene E., Research Assistant in Ophthalmology, College of Physicians and Surgeons
Lowenstein, Otto, Research Associate in Ophthalmology, College of Physicians and Surgeons
Malkiel, Saul, Research Associate, Harvard Medical School
McDonald, Sister Elizabeth Seton, Professor of Biology, College of Mt. St. Joseph
McIntire, F. C., Head of Biochemical Research, Abbott Laboratories
Novikoff, Alex B., Research Professor, Albert Einstein College of Medicine
Pullman, Bernard, Institut de Biologie Physico-Chimique, Paris
Reeves, Robert Blake, Assistant Professor of Zoology, Cornell University
Roth, Jay S., Professor of Biochemistry, University of Connecticut
Sandeen, Muriel I., Assistant Professor of Zoology, Duke University
Schlamowitz, Max, Associate Cancer Research Scientist, Roswell Park Memorial Institute
Spiegel, Melvin, Assistant Professor of Zoology, Dartmouth College
Uretz, Robert B., Assistant Professor of Biophysics, University of Chicago
Wainio, Walter W., Professor of Biochemistry, Rutgers, The State University
Wheeler, George E., Assistant Professor of Biology, Brooklyn College
Wilson, Irwin B., Associate Professor of Biochemistry, College of Physicians and Surgeons
Yntema, Chester L., Professor of Anatomy, State University of New York, Upstate Medical Center
Zorzoli, Anita, Associate Professor of Physiology, Vassar College

Students, 1961

All students listed completed formal course program, June 19 to July 29th. Asterisk indicates students completing Post Course Research Program, July 30 to September 2nd.

ECOLOGY

*Mary A. Ashcraft, Wilson College
*Robert J. Barsdate, University of Pittsburgh
Carol A. Baumann, Chatham College
*L. Lehr Brisbin, Wesleyan University
Charles H. Butterfield, Chevy Chase, Maryland
David A. Dobbins, University of Minnesota
*Roger W. Doyle, Dalhousie University
*Dirk Frankenberg, Emory University
*Thomas A. Gaucher, Narragansett Marine Laboratory
John Gutknecht, University of North Carolina
*William T. Hall, Fordham University
George Hayward, Washington University
Conrad A. Istock, University of Michigan
Marvin P. Kahl, University of Georgia
*Walter E. Knox, Drew University
Barbara March, Marquette University
*Elba L. Mas, Yale University
*Fermin Sagardia, Rutgers University
*Judith A. Shulman, Cornell University
*Sandra Elizabeth Wagner, Vassar College
*Margaret J. Waldrep, University of Alabama
Robert G. Wetzel, University of California

BOTANY

Marilyn L. Albert, University of Texas
George C. Carroll, Swarthmore College
*Charles F. Cleland, Wabash College
Eleanor Cox, University of Texas
*William H. Darden, Jr., University of Alabama
Victor Emanuel, University of Texas
*Robert B. Gordon, University of California
*Dana G. Griffin, III, Texas Technological College
*R. Don Groover, University of Alabama
*Jean Hatch, Vassar College
Raymond W. Holton, University of Michigan
Robert Hoshaw, University of Arizona
*Patricia Manco, Clark University
Robert McClary, Indiana University
John C. Messenger, Yale University
*William R. Rayburn, Washington University
Alvin Reeves, II, Indiana University
*Michael J. Wynne, Washington University
*Joanne R. Ziegler, Cornell University

PHYSIOLOGY

*Eric R. Bischoff, Washington University
Richard C. Blinkoff, Rockefeller Institute
Iain Bowman, National Institutes of Health
*John W. Brewer, Johns Hopkins University

Edward A. Brunner, Hahnemann Medical College
*Graciela C. Candelas, University of Puerto Rico
Eugenie J. Dubnau, Columbia University
Paul T. Englund, Rockefeller Institute
Howard L. Gillary, Oberlin College
*Alan Hooper, Oberlin College
*Edwin F. Humpal, Jr., University of Minnesota
*Anna E. Kammer, University of California
Alexander Keynan, Israel Institute for Biological Research
*Lawrence M. Lichtenstein, Johns Hopkins University
Lu-Ku Li, Princeton University
*Harry J. Mersmann, St. Louis University
*William M. Mitchell, Johns Hopkins University
*Thomas A. Murphy, Yale University
*Kenneth W. Perry, Jr., Syracuse University
*Jacob Lee Raab, University of Chicago
*Elizabeth Rittenhouse, University of Michigan
Harriette C. Schapiro, University of Miami
Joseph P. Senft, University of Buffalo
Martha R. Sheek, St. Louis University School of Medicine
Harry W. Taber, University of Rochester
Kenneth S. Warren, National Institutes of Health
Charles D. Yegian, University of California
C. Richard Zobel, Johns Hopkins University

EMBRYOLOGY

Allan L. Allenspach, Iowa State University
Alvin J. Clark, Columbia University
Rev. Richard T. Cleary, Johns Hopkins University
*Stiles D. Ezell, Jr., Bryn Mawr College
*Paul E. Fell, Stanford University
Ellen Fisher, Mt. Holyoke College
*Linda Garrick, Goucher College
Henry B. Garrison, Yale University
Luigi Giacometti, Brown University
Donald S. Gorman, Harvard University
*Bernice Grafstein, McGill University
*Jerome Gross, Massachusetts General Hospital
*John L. Kelland, Princeton University
Janos Lanyi, Harvard University
Harris I. Lehrer, Brandeis University
Mical E. Middaugh, University of Minnesota
*Fernando L. Renaud-Renaud, University of Chicago
*Gretchen Schabtach, Carnegie Institution of Washington
*Robert L. Searls, Brandeis University
*Sidney B. Simpson, Jr., Tulane University
*Richard G. Skalko, University of Florida

INVERTEBRATE ZOOLOGY

Evelyn Aliferis, University of Massachusetts
*Peter B. Armstrong, University of Rochester
*Roland H. Bagby, University of Illinois
Ruth R. Bennett, Tufts College
Shirley Brody, University of Rochester
*Alber H. Cass, Jr., Dartmouth College

Richard H. Colby, Massachusetts Institute of Technology
Albert J. Corkill, De Paul University
Michael W. Dix, Harvard University
Jack D. Donahue, Columbia University
Donna C. Emrich, Wilson College
*Hector R. Fernandez, University of Miami
*James H. Funston, Earlham College
Ray H. Gavin, Howard University
*Marilyn Goldsmith, Brown University
*V. Ann Hale, McGill University
Sister Mary A. Handy, University of Notre Dame
Herman B. Hartman, American University
Sister Mary C. Herold, Fordham University
Avis G. Hull, Drew University
Astrid Kodric, D'Youville College
Omer R. Larson, University of Minnesota
Arthur C. Lerner, Lafayette College
*Ellen M. Levine, Washington Square College
Winter P. Luckett, University of Missouri
*James S. McDaniel, University of Oklahoma
Joyce T. McKee, New York University
Robert Allen Menzies, University of Florida
Alice T. O'Malley, Clark University
Kay Eileen Saeger, University of Illinois
Howard A. Scalzi, Washington and Jefferson College
*Jack L. Schwade, Rice University
Dorothy S. Searle, Oberlin College
Philip J. Skehan, Jr., Syracuse University
*Helen Stout, Radcliffe College
Clarence E. Styron, Jr., Davidson College
William H. Talbot, Rockefeller Institute
Margaret W. Tryon, Wheaton College
Martha E. Welsh, Oberlin College
Robert S. Wilcox, University of Oklahoma

3. Fellowships and Scholarships, 1961

Lucretia Crocker Scholarship:
Eleanor Cox, Botany Course
Robert G. Wetzel, Ecology Course
The Merkel H. Jacobs Scholarship:
Edwin F. Humpal, Jr., Physiology Course
William Mitchell, Physiology Course
The Edwin Grant Conklin Memorial Scholarship:
Sidney B. Simpson, Jr., Embryology Course
The Emma Coote Drew Memorial Scholarship:
Tom Murphy, Physiology Course

4. Tabular View of Attendance 1957–1961

	1957	*1958*	*1959*	*1960*	*1961*
Investigators—Total	326	410	427	458	458
Independent	186	203	215	231	224
Under Instruction	23	39	45	42	32
Library Readers	42	54	51	50	49
Research Assistants	75	114	116	135	151

STUDENTS—TOTAL	139	138	134	122	130
Invertebrate Zoology	55	55	49	43	40
Embryology	27	22	23	20	21
Physiology	30	27	27	28	28
Botany	18	18	20	18	19
Ecology	9	16	15	13	22
TOTAL ATTENDANCE	465	548	561	580	586
Less persons represented as both investigator and student	3	5	4	2	1
INSTITUTIONS REPRESENTED—TOTAL	129	142	143	144	132
By investigators	94	110	98	83	107
By students	35	74	73	61	70
SCHOOLS AND ACADEMIES REPRESENTED					
By investigators	1	2	2	5	3
By students	5		12	2	—
FOREIGN INSTITUTIONS REPRESENTED	16	26	38	14	28
By investigators	11	20	29	11	21
By students	5	6	9	3	7

5. INSTITUTIONS REPRESENTED, 1961

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Albert Einstein College of Medicine
American University
Argonne National Laboratory
Arizona University
Brandeis University
Brooklyn College
Brown University
University of Buffalo
University of California
Canisius College
Carnegie Institution of Washington
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Duke University
D'Youville College
Earlham College
Emory University
University of Florida
Florida State University
Fordham University
University of Georgia
Goucher College
Hahnemann Medical School
Harvard University
Harvard University Medical School
Howard University
University of Illinois
Indiana University
Institute for Muscle Research
Iowa University
Iowa State University
Jackson College
Jefferson Medical College
Johns Hopkins University
University of Kansas
Kent State University
Lafayette College
Loyola College
Marquette University
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Massachusetts Institute of Technology
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University of Minnesota
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Muskingum College

University of New Hampshire
New York State University, College of Medicine at Syracuse
New York State University, College of Medicine at Brooklyn
New York University
New York University, Bellevue Medical Center
New York University School of Dentistry
New York University, Washington Square College
New York State Department of Health
North Carolina State College
University of North Carolina
Northwestern University
Notre Dame University
Oberlin College
Ohio State University
University of Oklahoma
University of Pennsylvania
Pennsylvania University Medical School
University of Pittsburgh Medical School
Princeton University
Purdue University
Queens College
Radcliffe College
Reed College
University of Rhode Island
Rice University
University of Rochester
Rockefeller Institute
Roswell Park Memorial Institute
Russell Sage College
Rutgers University
St. John Fisher College
St. Louis University
St. Peter's College
Seton Hill College
Single Cell Research Foundation
Sloan-Kettering Institute
Smith College
Stanford University
Swarthmore College
Syracuse University
Temple University
Texas Technology College
Tufts University
Tulane University
U. S. Fish and Wildlife Service
U. S. Public Health Service
Vassar College
Veterans Administration Hospital at Albany
Veterans Administration Hospital at Brooklyn
University of Vermont
University of Virgina
Wabash College
Washington University
Washington University Medical School
Washington and Jefferson College
Wayne State University
Wesleyan University
Western Reserve University
Wilson College
Woods Hole Oceanographic Institution
Yale University

Foreign Institutions Represented

University of Puerto Rico
Dalhousie University, Canada
McGill University, Canada
Israel Institute for Biological Research
National Institute for Medical Research, London
Technilogical University, Delft, Netherlands
Institut for Sporeplanter, Copenhagen
University of the Philippines
University of Geneva, Switzerland
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6. FRIDAY EVENING LECTURES, 1961

June 30
C. R. AUSTIN .... "Variety in mammalian egg nuclei"

July 7
DANIEL E. KOSHLAND .... "Protein structure and enzyme specificity"

July 14
J. Z. YOUNG .... "The visual and statocyst systems of cephalopods"

July 21
J. Z. YOUNG .... "The learning systems of Octopus"

July 28
BENJAMIN ZWEIFACH .... "Reticulo-endothelial system in relation to adaptive reactions"

August 4
DAVID H. HUBEL .... "The eyes, the brain and perception"

August 11
ETIENNE WOLFF .... "Some aspects of the principle of competition in the developing limb of the chick embryo"

August 18
EDWIN CHARGAFF .... "Sequence problems in the deoxyribonucleic acids"

August 25
WILLIAM P. JACOBS .... "Compensatory growth, cell differentiation and flowering as analyzed with the aid of formal rules of proof"

7. TUESDAY EVENING SEMINARS, 1961

July 11	ROGER MILKMAN	"Temperature adaptation in Drosophila pupae"
	LIONEL I. REBHUN	"Endoplasmic reticulum in aster formation"
	DONALD P. COSTELLO	"The orientation of centrioles in dividing cells and its significance"
July 18	RUSSELL DOOLITTLE	"The nature of lamprey eel fibrinopeptide material"
	R. H. CHENEY	"Equivalent dosage effects of ultraviolet and x-ray irradiation of Arbacia gametes, as recorded by cinephotomicrography"
	C. C. SPEIDEL	"Time-lapse cinephotomicrographs illustrating abnormalities of viscosity, density, and cleavage in developing sea urchins derived from various fertilization combinations of irradiated gametes"

July 25	A. Kent Christensen	"Fine structure of an unusual sperm in the flatworm Plagiostomum"
	Alex B. Novikoff	"Observations on the golgi apparatus and related lysosomes"
	Berta Scharrer	"Functional analysis of the corpus allatum of the insect, Leucophaea maderae, with the electron microscope"
August 8	Eugene Copeland	"Ultrastructure of teleost swim bladder"
	Philip B. Dunham	"The physiological basis of acclimation of Tetrahymena to high NaCl medium"
	Eugene P. Odum	"Excretion rate of radio-isotopes as indices of metabolic rates in nature: biological half-life of zinc-65 in relation to food consumption, growth and reproduction in arthropods"
August 15	Paul Weiss	"Motion picture records of cell interactions: responses of different cell types to medium, to substratum, and to each other"
August 22	Emilienne Wolff Etienne Wolff	"In vitro culture of human tumors on explants of chick embryonic organs"

8. Members of the Corporation, 1961

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V. REPORT OF THE LIBRARIAN

In 1961, the Library received 1730 current journals, 59 new titles having been added during the year. Of the total, the Marine Biological Laboratory subscribed to 509, received 648 in exchange and 192 as gifts. The Woods Hole Oceanographic Institution subscribed to 115, received 204 in exchange and 62 as gifts.

The Laboratory purchased 63 books, received 109 complimentary copies (8 from authors and 101 from publishers) and accepted 62 miscellaneous gifts. The Institution purchased 67 books and received 22 as gifts. The total number of books accessioned totalled 323.

Through purchase, exchange, and gift, the Laboratory completed 16 journal sets and partially completed 25. The Institution completed 7 sets and partially completed 8. There were 2647 reprints added to the collection, of which 1809 were of current issue.

The Library now contains 78,800 bound volumes and 219,099 reprints. There were 535 journal volumes sent out on interlibrary loan and 49 borrowed. About 1200 volumes were bound.

Books and reprints were presented by Drs. Irvine H. Page, John P. Hervey, Kirk Bryan and Roberts Rugh. A set of the Catholic Encyclopedia was given by St. Joseph's College. The Library extends grateful acknowledgment to these generous donors.

In September the Library purchased a photocopying machine. This is for use in supplying (at cost) short articles requested on interlibrary loan. This service eliminates sending out valuable publications and decreases the wear and tear brought about by interlibrary loans. From September through December, 52 requests were filled by photoprints.

The circulation of books and reprints increased greatly, thus emphasizing the trend of growth established during the last few years. The Library facilities are now available, throughout the winter months, to local doctors and to college and high school students. Expansion and improvements are now being planned and the staff looks forward to a year of progress.

Respectfully submitted,
Deborah L. Harlow,
Librarian

VI. REPORT OF THE TREASURER

The market value of the General Endowment Fund and the Library Fund at December 31, 1961, amounted to $2,025,139 as against book value of $1,193,853. This compares with values of $1,796,571 and $1,146,393 at the end of the preceding year. The average yield on the securities was 3.37% of the market value and 5.72% of book value. The total uninvested principal cash in the above accounts as of December 31, 1961, was $1,932. Classification of the Securities held in the Endowment Funds appears in the Auditor's report.

The market value of the pooled securities as of December 31, 1961, was $373,641 with uninvested principal cash of $283.27; the market value at December 31, 1960 being $333,218. The book value of the securities in this account was $293,068 on December 31, 1961, compared with $274,294 a year earlier. The average yield on market value was 3.39% and 4.32% of book value.

The proportionate interest in the Pool Fund Account of the various Funds as of December 31, 1961, is as follows:

Pension Funds	26.109%
General Laboratory Investment	52.177
Other:	
Bio Club Scholarship Fund	1.494
Rev. Arsenius Boyer Scholarship Fund	1.829
Gary N. Calkins Fund	1.711
Allen R. Memhard Fund	.332
F. R. Lillie Memorial Fund	5.766
Lucretia Crocker Fund	6.243
E. G. Conklin Fund	1.056
M. H. Jacobs Scholarship Fund	.753
Jewett Memorial Fund	.557
Anonymous Gift	1.973

Donations from the M.B.L. Associates for 1961 were $4,330 as compared with $4,320 for 1960. Unrestricted gifts from foundations, societies and companies amounted to $36,348.

The James Foundation made a gift of $50,000 towards the building of additional housing units in Devil's Lane which will be ready for occupancy in June, 1962.

We are administering 15 grants for investigators in addition to those grants made directly to the Marine Biological Laboratory for general support and training courses. The amounts of grants vary in accordance with the investigator's project of research. An amount of 15% based on amount expended is allowed the Laboratory as overhead.

The Lillie Fellowship Fund with a market value of $96,705 and a book value of $92,464, as well as the investment in the General Biological Supply House with a book value of $12,700, is carried in the Balance Sheet, item "Other Investments." The General Biological Supply House for the fiscal year ended June 30, 1961, had a profit after taxes of $312,000 as compared to $314,034 in 1960 and $303,300 in 1959 and $218,210 in 1958, and $123.430 in 1957. In the fiscal year 1961, the Marine Biological Laboratory received dividends from the General Biological Supply House of $33,020 as against $30,480 in 1960 and $30,480 in 1959 and $25,400 in 1958.

Following is a statement of the auditors:

To the Trustees of the Marine Biological Laboratory, Woods Hole, Massachusetts:

We have examined the balance sheets of the Marine Biological Laboratory as of December 31, 1961 and 1960, the related statements of operation expenditures, income and current fund for the years then ended, and statement of funds for the year ended December 31, 1961. Our examination was made in accordance with generally accepted auditing standards, and accordingly included such tests of the account records and such other auditing procedures as we considered necessary in the circumstances.

In our opinion, the accompanying financial statements present fairly the assets, liabilities and funds of the Marine Biological Laboratory at December 31, 1961, and the results of its operations for the year then ended.

Boston, Massachusetts
March 16, 1962

LYBRAND, ROSS BROS. & MONTGOMERY

JAMES H. WICKERSHAM,
Treasurer

MARINE BIOLOGICAL LABORATORY

BALANCE SHEETS

December 31, 1961 and 1960

Investments

	1961	*1960*
Investments held by Trustee:		
Securities, at cost (approximate market quotation 1961—$2,025,000)	$1,193,853	$1,146,393
Cash	1,932	255
	1,195,785	1,146,648
Investments of other endowment and unrestricted funds:		
Pooled investments, at cost (approximate market quotation 1961—$373,641) less $5,728 temporary investment of current fund cash	287,340	268,566
Other investments	138,546	137,742
Cash	12,764	10,839
Accounts receivable	41	21
	$1,634,476	$1,563,816

Plant Assets

Land, buildings, library and equipment (note)	4,795,960	3,280,059
Less allowance for depreciation (note)	1,189,121	1,142,879
	3,606,839	2,137,180
Construction in progress	105	1,455,811
Cash		82,042
U. S. Government obligations, at cost:		
$50,000 Treasury certificates, due 5/15/62	50,000	
	$3,656,944	$3.675,033

Current Assets

Cash	65,623	77,546
Temporary investment in pooled securities	5,728	5,728
Accounts receivable (U. S. Government, 1961—$20,129; 1960—$43,443)	49,290	59,889
Inventories of specimens and Bulletins	43,712	47,641
Prepaid insurance and other	6,870	16,778
	$ 171,223	$ 207,582

MARINE BIOLOGICAL LABORATORY

Balance Sheets

December 31, 1961 and 1960

Endowment Funds

	1961	*1960*
Endowment funds given in trust for benefit of the Marine Biological Laboratory	$1,195,785	$1,146,648
Endowment funds for awards and scholarships:		
Principal	126,302	126,302
Unexpended income	9,600	7,285
	135,902	133,587
Unrestricted funds functioning as endowment	206,378	206,378
Retirement fund	81,790	71,449
Pooled investments—accumulated gain	14,621	5,754
	$1,634,476	$1,563,816

Plant Liability and Funds

Funds expended for plant, less retirements	4,796,065	4,668,475
Less allowance for depreciation charged thereto	1,189,121	1,142,879
	3,606,944	3,525,596
Unexpended plant funds	50,000	82,042
	3,656,944	3,607,638
Accounts payable		67,395
	$3,656,944	$3,675,033

Current Liabilities and Funds

Accounts payable	30,337	41,106
Unexpended research grants	52,837	51,726
Unexpended balances of gifts for designated purposes	8,878	9,663
Current fund	79,171	105,087
	$ 171,223	$ 207,582

Note—The Laboratory has since January 1, 1916, provided for reduction of book amounts of plant assets and funds invested in plant at annual rates ranging from 1% to 5% of the original cost of the assets.

MARINE BIOLOGICAL LABORATORY

Statements of Operating Expenditures, Income and Current Fund

Years Ended December 31, 1961 and 1960

Operating Expenditures

	1961	*1960*
Research and accessory services	$ 268,193	$ 250,578
Instruction	158,780	219,234
Library and publications	67,189	61,462
Direct costs on research grants	177,938	182,899
	672,100	714,173
Administration and general	83,366	70,037
Plant operation and maintenance	118,993	117,980
Dormitories and dining	160,838	162,713
Additions to plant from current income	27,210	78,654
	1,062,507	1,143,557
Less depreciation included in plant operation and dormitories and dining above but charged to plant funds	48,419	48,086
	1,014,088	1,095,471

Income

	1961	*1960*
Research fees	102,081	56,408
Accessory services (including sales of biological specimens, 1961—$43,045, 1960—$48,817)	86,790	151,109
Instruction fees	27,730	23,905
Grants for instruction and research training	148,078	185,571
Library fees, Bulletin subscriptions and other	39,575	35,174
Reimbursements and allowances for direct and indirect costs on research grants	195,716	221,197
Dormitories and dining income	123,231	105,086
	723,201	778,450
Gifts used for current expenses	40,468	48,300
Grants used for current expenses	114,170	143,018
Investment income used for current expenses	110,333	105,066
Total current income	988,172	1,074,834
Excess of operating expenditures over current income	25,916	20,637
Current fund balance January 1	105,087	125,724
Current fund balance December 31	$ 79,171	$ 105,087

MARINE BIOLOGICAL LABORATORY

Statement of Funds

Year Ended December 31, 1961

	Balance Jan. 1, 1961	*Gifts and Other Receipts*	*Investment Income*	*Used for Current Expenses*	*Other Expenditures*	*Balance Dec. 31, 1961*
Invested funds	$1,563,816	$ 76,274	$116,909	$108,863	$ 13,660	$1,634,476
Unexpended plant funds	$ 82,042	63,290			95,332	$ 50,000
Unexpended research grants	$ 51,726	468,319		467,208		$ 52,837
Unexpended gifts for designated purposes	$ 9,663	40,678		40,468	995	$ 8,878
Current fund	$ 105,087			25,916		$ 79,171
		$648,561	$116,909	$642,455	$109,987	
Gifts		103,968				
Grants for research, training and support		468,319				
Net gain on sales of securities		58,004				
Appropriated from current income and other		18,270				
		$648,561				
Expended for construction of new building					95,332	
Scholarship award					2,700	
Payments to pensioners					10,960	
Other					995	
					$109,987	

MARINE BIOLOGICAL LABORATORY

Summary of Investments of Endowment Funds

December 31, 1961

	Cost	% of Total	Market Quotations	% of Total	Investment Income 1961
Securities held by Trustee:					
General endowment fund:					
U. S. Government securities	$ 35,110	3.6	$ 35,963	2.1	$ 1,651
Corporate bonds	514,520	52.0	501,318	29.8	$ 20,625
Preferred stocks	84,778	8.6	73,012	4.4	3,283
Common stocks	354,366	35.8	1,069,462	63.7	31,399
	988,774	100.0	1,679,755	100.0	56,958
General Educational Board endowment fund:					
U. S. Government securities	31,040	15.1	31,853	9.2	1,491
Other bonds	86,679	42.3	87,550	25.3	3,125
Preferred stocks	26,745	13.0	25,312	7.3	1,160
Common stocks	60,615	29.6	200,669	58.2	5,649
	205,079	100.0	345,384	100.0	11,425
Total securities held by Trustee	$1,193,853		$2,025,139		$ 68,383
Investments of other endowment and unrestricted funds:					
Pooled investments:					
U. S. Government securities	15,111	5.2	15,027	4.0	146
Corporate bonds	134,718	46.0	134,474	36.0	6,928
Preferred stocks					192
Common stocks	143,239	48.8	224,140	60.0	5,408
	$ 293,068	100.0	$ 373,641	100.0	$ 12,674
Other investments:					
U. S. Government securities	$ 7,000				$ 350
Other bonds	47,906				1,998
Preferred stocks	3,728				131
Common stocks	46,530				34,195
Real estate	33,382				
	138,546				36,674
Total investments of other endowment and unrestricted funds	$ 431,614				$ 49,348
Total investment income					$117,731
Custodian's fees charged thereto					(577)
Income of current funds temporarily invested in pooled securities					(245)
Investment income distributed to funds ...					$116,909

MARINE BIOLOGICAL LABORATORY
LIBRARY
WOODS HOLE, MASS.

MATING BEHAVIOR AND SOCIAL STRUCTURE IN LOLIGO PEALII

JOHN M. ARNOLD

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In the course of studies on embryological development of the squid, *Loligo pealii,* observations of the social behavior and mating reactions of this species were made. Observations made in the laboratory were confirmed by watching squid in their natural habitat. These studies have revealed a method for artificially stimulating mating and subsequent egg-laying. It is thus possible to have a reliable source of squid eggs, at convenient times, in the laboratory. Drew (1911) made extensive observations on copulation and egg-laying in *Loligo pealii* but, to the author's knowledge, nothing has been recorded on mate selection and social structure in this species.

Squid are particularly suitable for a study of social behavior for a number of reasons. They are pelagic and gregarious. Since there are no territorial separations, there is ample opportunity for a maximum of intraspecific interaction and, therefore, for the development of a social structure. They possess a moderate amount of intelligence which would enhance the establishment of social structure. Other than a few scattered observations on the Octopoda, very little has been published on the social behavior of the Cephalopoda. This paper is an attempt to show that *Loligo pealii* has a social structure concerned with its mating behavior.

Materials and Methods

The animals used in this study were captured in fish traps in the vicinity of Woods Hole or Barnstable, Massachusetts, in the summers of 1960 and 1961. Late in the summer of 1961 some animals were used that had been captured in fish traps off the coast of Rhode Island. There seemed to be some minor differences in these two populations, indicated by their interaction, but their intra-population interaction was the same. The animals were all sexually mature but varied in mantle length from about six to ten inches. The males appeared to be slightly larger than the females. The animals were obtained from the Supply Department of the Marine Biological Laboratory, usually shortly after their capture and at most one day thereafter. They were kept in a 2½ by 6 foot fiberglass tank filled to a depth of 12 inches with rapidly running sea water. Usually from four to ten animals were kept in such a tank at one time without apparent overcrowding. Usually more males were present than females, although in some cases the situation was reversed. The animals were fed about two *Fundulus heteroclitus* per squid, usually daily. Under these conditions the squid survived about five days. In one case a male survived for seventeen days in a tank that was undisturbed except for periodic feeding.

Males and females are easily distinguished by the presence of the white testis in the male, the slimmer outline of the males, and the presence of the bright orange accessory nidamental gland visible through the mantle of the female. Individuals of either sex could be distinguished by their size or by characteristic wounds and scars on their mantles.

The animals were stimulated with a naturally laid egg mass tied to a cotton cord and lowered into one corner of the tank. An artificial egg mass was constructed of water-filled tubing made of polyethylene sheet fused together at the edges. A small amount of phenol red was added to the water used in the tubing to give it an orange color like that of the egg mass. Other animals were introduced to an existing group by slowly submerging a bucket containing the new animals into the tank and allowing them to swim out. The observations of squid in nature were made from a fixed wharf or an anchored boat in shallow water after dark. These observations were made with the assistance of a fixed incandescent lamp.

OBSERVATIONS

Initiation of sexual behavior

Normally, the captive squid swim parallel to each other in a small school moving back and forth in synchrony. There is no apparent social order to their position in these small schools. The males and females move about in the tanks paying no apparent attention to each other. Since these animals were chosen randomly from a much larger group (about 30–50), any prior social pattern is assumed to have been broken. There seems to be no particular dominance in feeding behavior since any *Fundulus* offered is taken by the nearest squid. No other evidence of any social pattern has been observed and if any such structure existed at this time it was latent. Therefore, it is assumed that mate selection had not taken place in this condition. In these circumstances a stimulus could be presented and characteristic responses observed.

If a naturally laid egg mass was tied to a string and placed in one corner of the tank, the squid almost immediately "broke formation" and investigated the egg mass. This response began when the animals swam rapidly toward the egg mass, formed their arms into a cone, and pointed at the egg mass. Occasionally the egg mass would be flushed with spurts of water from the funnel of an approaching squid. One animal after another would dart up to the egg mass and "feel" it with its arms. Then each would rapidly dart away and rejoin the group. Two individuals would occasionally approach the egg mass at the same time. Both males and females would show this response which would occur within about twenty seconds after the introduction of the egg mass.

This stimulus seemed to be completely visual because of the speed with which the squid responded. This hypothesis was checked by using an artificial egg mass of polyethylene tubing. This artificial egg mass elicited the same response as a real egg mass. The animals investigated and felt it but responded abnormally by flushing it repeatedly with spurts of water from their funnels and by swimming excitedly to and fro. However, this was still followed by normal mate selection and the establishment of a hierarchy. Usually the egg string would be held in the arms for several minutes and then dropped on the bottom of the tank. In two cases egg capsules were actually laid on the artificial egg mass although the female made several approaches before finally attaching the egg string. Further evidence of the visual nature of this stimulus can be drawn from the fact that in the absence of an egg mass, egg strings would be attached to anything that resembled an egg mass. Several times egg strings have been deposited on the arms of a dead squid left in the tank. In cases of deprivation of stimulus the squid will even investigate the extended fingers of a human hand placed in the tank (not recommended).

The stimulus worked best on squid that had not been known to have bred recently, hence the necessity of getting the animals as soon as possible after their capture. If deprived of stimulus for a long period of time, females would eventually drop egg strings but made no apparent attempt to form an egg mass unless a nucleus of egg strings accumulated by chance at the tank drain.

This response to an egg mass has been observed in nature by dangling an egg mass on a string in front of a school of squid. Egg-laying has occurred in these egg masses.

Establishment of a hierarchy

The investigative behavior was followed by dominance determination behavior. This began by the males raising one median arm a few centimeters above the rest of the arms and waving it. Sham battles usually followed in which the males rushed at each other but did not actually touch each other. At the same time they developed dark brown lateral areas at the base of the arms. This color pattern seemed to be characteristic of sexually aroused male squid. During this time a given male would place himself between the rest of the group and a female of his choice. Any approaching male would be threatened by a waving of the median arm. If the approaching male was persistent, he would be driven off by rushes from the selecting male. In cases where the intruder was extremely insistent, the two males would sometimes bump tails and display characteristic dark colored spots along the lateral margins of the fins. These spots were not observed at any other time on any male. This has been interpreted as a further warning sign. In a relatively few cases there was actual contact between two males. In these cases the males rushed at each other and one grasped the other around the mantle. In three observed cases the arms interlocked and in one case the tip of an arm was bitten off. This behavior continued between all the males in the tank until one male was established as the dominant male. Other males appeared subordinate to this male. The subordinate males each selected a female and would undergo similar combat among themselves. An individual's position in the social structure seemed roughly correlated with its size. Since there were fewer females than males in the tank, a few males did not have mates. Only rarely did one of the mateless males succeed in displacing a mated male. The squid taken from the traps at Rhode Island seemed to be more aggressive and would challenge and displace larger squid taken from the Cape Cod population. As the physical conditions of a male deteriorated the same social structure remained until he reached a completely defenseless state and another male could take over his female. If a new male was introduced to a group that had established a social structure he would be immediately challenged by the other males until he was integrated into the social structure. This would often result in a displacement of an established male and the displaced male would then displace his subordinate or, if in poor physical condition, be relegated to the mateless group. Only rarely did any male attempt to change its mate spontaneously.

The behavior of the females was less active. Normally a female would show slight avoidance of a male but seemed to have no mate preference. While mate selection and challenging went on between males, females paid no apparent attention to the males. Once the social structure among males had been established the males attempted copulation with the females. Normally a female would resist by darting away quickly when the male attempted copulation. After a short interval the female usually accepted the male's advances and copulation followed.

Copulation and egg-laying

The behavior and events during copulation have been well described by Drew (1911) and only details not recorded there will be mentioned here. The male swam parallel to the female and moved back and forth exactly at the same time (assuming no other male intruded). The spots at the base of the arms of the male became intense. Occasionally copulation was preceded by the male reaching out with one arm and lightly touching the female on the mantle behind the head. There were two methods of copulation observed. Most common was the lateral method in which the male paralleled the female and grasped her around the mantle behind the head, the hectocotylized arm was placed into his own mantle, spermatophores picked up, and then transferred to the female by way of her funnel. This took about ten seconds. The second method of copulation involved a meeting of the male and female head to head and probably resulted in a transfer of the spermatophores to the buccal pouch. This method was infrequently observed.

Occasionally a female would strongly resist a male and would not permit copulation at all. This was usually done by swimming rapidly away or by struggling when the male grasped her. Rarely, if a male was very aggressive, the female would actively resist by grasping the male with her arms. On three occasions females have propelled themselves out of the tank in an attempt to escape an aggressive male. In cases where the females outnumbered the males a polygamous relationship would develop. In two cases, when males were in reduced physical condition, the females took the aggressive role and attempted to force copulation by grasping the male about the mantle. In one case an aggressive female could get no response and finally ate part of the male. Several times males displayed necrophilia (Daveian behavior) when no living females were available to them.

Copulation was usually followed by egg-laying. Drew (1911) has given an elaborate account of egg-laying and all that will be mentioned here is a brief account for the sake of completeness. After copulation the female approached the egg mass and again investigated it. An egg string was then passed from the funnel to the arms which encircled it. The female approached the egg mass and reached into the center and manipulated the egg string into place. One end of the egg string is free of eggs, narrower, and denser in composition. This end was intertwined in the egg mass with the tips of the arms so that the string was firmly enmeshed. If during this time the string was dropped the complete operation was continued as if nothing abnormal had happened. The female quickly retreated from the egg mass following the attachment of the egg string. A new egg string was then passed up to the arms and the process repeated. In one case, seven females added 26 egg strings to one small egg mass of twenty strings in thirteen minutes.

Discussion

The major points of this paper are the nature of the stimulus of reproductive behavior and the resultant social structure. The stimulus was no doubt visual because of the speed at which the response occurred and the fact that objects having a resemblance to egg masses would cause such a response. It is the author's opinion that this stimulus elicits sexual behavior. This would explain the fact that egg masses are often found attached to *Fucus* or similar sea weeds. Several females deposit their egg strings on a common egg mass which agrees with this hypothesis. The report of vast beds of squid eggs off the California coast could also be explained by such a hypothesis (McGowan, 1954). Undoubtedly, there must be another stimulus that elicits the original mating that results in the formation of the original egg mass. This egg mass then elicits mating behavior in other squid. The nature of the original stimulus remains unknown and it is possible that this original mating occurs spontaneously.

The social structure in the males appears to be a classical peck order with an establishment of a dominant male able to resist all other males, and of a series of subordinate males. These subordinate males are able to resist males of a lower position but may be displaced by a male of higher position.

The author wishes to thank Mr. Robert O. Lehy of the Marine Biological Laboratory Supply Department for his cooperation with this project, and Dr. N. T. Spratt, Dr. R. K. Josephson, and Mr. R. B. Forbes for reading and criticizing this manuscript.

Summary

1. Observations of *Loligo pealii* have shown the egg mass can stimulate sexual behavior. This stimulus apparently has a visual basis.

2. This stimulus is followed by establishment of a social hierarchy and by mate selection by the males. The males exhibit warning displays, sham battles, and mate protection during this time.

3. Normally the females respond passively but occasionally they will take an aggressive role.

4. This mating behavior results in copulation and egg-laying; thus a method for obtaining naturally laid eggs has been revealed.

LITERATURE CITED

Drew, G. A., 1911. Sexual activities of the squid *Loligo pealii.* I. *J. Morph.,* **22**: 327–360.

McGowan, J. A., 1954. Observations on the sexual behavior and spawning of the squid *Loligo opalescens* at La Jolla, California. *Calif. Fish. Game,* **40**: 47–54.

RESPIRATION, ELECTRON–TRANSPORT ENZYMES, AND KREBS–CYCLE ENZYMES IN EARLY DEVELOPMENTAL STAGES OF THE OYSTER CRASSOSTREA VIRGINICA[1]

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A marked increase in the rate of respiration during early development has been noted in many different organisms (see Brachet, 1950; Boell, 1955). In most cases the changes in respiratory enzymes which might contribute to the respiratory increases have not been fully studied. Of particular interest in this connection are studies which have dealt with enzymes of the tricarboxylic acid (citric acid) cycle, as well as those investigations of the enzymes involved in the transfer of electrons between substrates and oxygen, via the systems which oxidize reduced pyridine nucleotides and succinic acid.

Parallels between the increase in respiration and that of cytochrome oxidase have been noted in the grasshopper (Bodine and Boell, 1936; Allen, 1940), the salamander (Boell, 1945), and the chick (Albaum and Worley, 1942; Albaum *et al.*, 1946; Levy and Young, 1948). In *Xenopus laevis* Boell and Weber (1955) have reported an increase in cytochrome oxidase beginning during cleavage. This is in contrast to the data of Spiegelman and Steinbach (1945) on the developing eggs of *Rana pipiens* and to those of Petrucci (1957) on cytochrome oxidase during the early development of *Bufo bufo*. In the developing salamander increases in the activity of succinoxidase have been shown to be similar to those of cytochrome oxidase (Boell, 1948; Krugelis *et al.*, 1952).

Several enzymes of the tricarboxylic acid cycle have been studied in embryos of the chick between the ages of 2 and 6½ days of incubation by Mahler, Wittenberger and Brand (1958). Aconitase, isocitric dehydrogenase, alpha-keto glutaric dehydrogenase, succinic dehydrogenase, malic dehydrogenase, and fumarase all increased at rates equal to or above that of total embryonic protein up to three days of incubation; following this time only malic dehydrogenase was found to accumulate as rapidly as total protein. On the basis of assays in homogenates these authors concluded that the oxidation of pyruvate by enzymes of the citric acid cycle is probably not the rate-limiting factor in the embryonic respiration. Brand and Mahler (1959) have obtained similar results in the chick in an investigation of enzymes oxidizing reduced diphosphopyridine nucleotide. Diaphorase, DPNH-oxidase, and cytochrome oxidase all increased in specific activity until the fourth day of development, after which their rate of accumulation was exceeded by that of other embryonic protein.

In eggs of marine invertebrate animals few studies have been made of changes in respiratory enzymes during development. In the sea urchin Gustafson and Hasselberg (1951) found that the activities of succinic dehydrogenase and malic dehydrogenase increased after the mesenchyme-blastula stage. Deutsch and Gustafson (1952) found lower activities of cytochrome oxidase in homogenates of blastulae than in those of cleaving eggs. In an attempt to relate respiratory changes to changes in enzymes, Runnström (1930, 1956) has postulated that the terminal oxidase system is present but relatively inactive until the swimming, larval stage is reached in the sea urchin. Black and Tyler (1959) have reached a similar conclusion for eggs of *Urechis caupo* and *Strongylocentrotus purpuratus* on the basis of relative rates of oxidation of carbon monoxide in the light and dark by these developing embryos.

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[1] This investigation was supported by a grant (G-9847) from the National Science Foundation.

[2] Contribution No. 116.

In view of the importance of enzymes of the citric acid cycle and the terminal electron-transport system in the respiration of most animals, it is of interest to extend the present knowledge concerning developmental changes in these enzymes to embryos of marine invertebrate animals other than the sea urchin. In the present report, therefore, data on changes in the respiration and in the activities of several respiratory enzymes of early embryos of the oyster, *Crassostrea virginica,* are presented. Cleland (1951) has demonstrated the presence of most enzymes of the Krebs citric acid cycle in homogenates of oyster eggs, since he obtained respiratory stimulation in such homogenates after the addition of intermediate substrates of this cycle. In addition, succinoxidase and cytochrome oxidase have been shown to be present in the same material by Cleland. The present study is the first in which the existence of a DPNH-oxidase system has been demonstrated and in which direct methods have been used to indicate the presence of enzymes of the Krebs cycle in oyster embryos.

Materials and Methods

Oysters were collected during the spawning season from pilings at Gloucester Point, Virginia, and they were stored in a refrigerated river-water system, in which the salinity was 18 to 20 ppm. To obtain gametes, the animals were opened, and portions of the gonads were removed and shaken gently in the water. The eggs were washed by settling and inseminated. The fertilized eggs were washed several times by settling until excess sperm had been removed. The eggs were cultured in a rotating flask at 20° C. in water of the above salinity containing 0.005 M glycylglycine buffered to pH 7.8. Penicillin (100 units per ml.) and streptomycin (50 micrograms per ml.) were added to all cultures to retard bacterial growth. The concentration of eggs in the rotating flask never exceeded 10^7 per liter.

Since it was difficult to ascertain the percentage of fertilization immediately, eggs were removed for the first measurements of respiration and/or enzyme activity immediately after the first cleavage (about 1–1½ hours following fertilization). In all experiments reported the percentage of cleaving eggs was better than 85%. Later times at 20° C. and stages used for subsequent measurements were as follows: 8–10 hours (swimming blastula), 23–25 hours (trochophore), and 48–50 hours (early veliger). Only swimming embryos were used for measurements in blastula, trochophore, and veliger stages.

The eggs or embryos removed for analysis were washed by centrifugation and suspended in a known volume of sea water (25–50 ml.), so that aliquots could be taken for counting. From the suspension, five aliquots of 0.5 or 1.0 ml. were taken, and these were each diluted to 25 or 50 ml. From each diluted aliquot two samples of 0.5 or 1.0 ml. were counted, making a total of ten counts (1,000 to 2,000 embryos) in all.

In one series of experiments measurements of respiration were made on living embryos and they were then homogenized in dilute phosphate buffer for measurements of succinic dehydrogenase, DPNH-oxidase, and cytochrome oxidase. For measurements of respiration the embryos were suspended in dilute sea water containing 0.005 *M* glycylglycine, pH 7.8, plus penicillin and streptomycin as indicated above. Aliquots of 2.0 ml. were measured into duplicate Warburg vessels of 15-ml. capacity and respiration was measured at 25° C. for one hour. The embryos were then rinsed carefully into centrifuge tubes, packed by centrifugation, resuspended in 10 volumes of cold 0.033 *M* phosphate, pH 7.4, and homogenized with a syringe and No. 20 needle. The homogenate was made to a known volume (1 to 5 ml.) in a calibrated vessel, and aliquots were withdrawn for the determination of the activities of cytochrome oxidase, DPNH-oxidase, and succinic dehydrogenase. Measurements of these activities were completed within 1½ hours after homogenization.

In other series of experiments measurements of the activities of TPN-specific isocitric dehydrogenase and alpha-ketoglutaric dehydrogenase and of malic dehydrogenase and aconitase were performed. For assaying the first two enzymes, embryos were homogenized in a solution containing 0.3 *M* sucrose and 0.05 *M* Tris (hydroxymethyl) aminomethane, pH 7.4. The latter two enzymes were assayed in homogenates made in 0.03 *M* Tris, pH 7.4. All measurements were completed within 1½ hours after homogenization.

All of the measurements of enzyme activity were performed at 25° C. With the exception of cytochrome oxidase, which was assayed manometrically, the activities of all enzymes were determined by spectrophotometric methods. For the latter assays, 3.0-ml. silica cuvettes of 1-cm. light path were used in a Beckman Model DU spectrophotometer. Between readings the cuvettes were incubated in a water bath at 25° C. or at room temperature of 25°.

All assays were performed at substrate concentrations which allowed initial rates to follow zero-order kinetics. Assays were always performed at two or more levels of homogenate concentration. This provided continuous assurance that reaction rates were directly proportional to the concentration of homogenate in the reaction mixtures. In general, duplicate determinations were made at one concentration of homogenate and a single determination was made at half this concentration. Analyses in which the reaction rate was not proportional to homogenate-concentration or in which the rate of reaction was not constant were discarded. Differences between rates recorded for any pair of duplicates seldom exceeded 10% of the mean rate for the pair. Details of the assay methods for the enzymes are listed below. (Homogenate percentages given below are approximate, based on volumes of packed embryos.)

Cytochrome oxidase. Manometric method of Schneider and Potter (1943). Warburg vessels contained 0.02 *M* ascorbic acid, pH 7.4, 2×10^{-4} *M* cytochrome *c* (based on M. W. of 16,000), 0.067 *M* phosphate, pH 7.4, 6×10^{-4} *M* aluminum chloride, and 0.5 to 1.5 ml. of 5% homogenate. Assays were made at three levels of homogenate concentration, and the auto-oxidation rates of ascorbic acid were obtained by extrapolating the rates of oxygen uptake to zero homogenate concentration. After equilibration, readings were taken for 5–10 minutes before tipping in 2×10^{-3} *M* cytochrome *c* from the side arm. The initial rate of activity was calculated on the basis of three readings taken at 5-minute intervals after the addition of cytochrome *c* from the side arm. The endogenous oxygen uptake was obtained by subtracting the low rate of auto-oxidation of ascorbic acid in the absence of cytochrome *c* from the rate of uptake in the vessels before the cytochrome *c* was added to the main chamber. The endogenous rates of oxygen uptake were usually less than 5% of the rates in the presence of cytochrome *c*. The auto-oxidation rates of ascorbic acid when both cytochrome *c* and homogenate were present varied between 60 and 90 microliters per hour in different experiments.

DPNH-oxidase. Spectrophotometric method of Brand and Mahler (1959). The cuvettes contained 1.7×10^{-4} *M* reduced diphosphopyridine nucleotide (DPNH), 3×10^{-6} *M* cytochrome *c*, 0.05 *M* phosphate, pH 7.4, and 0.05 to 0.2 ml. of 10% homogenate. The blank cuvette contained all components except homogenate. After the addition of all substances to blank and experimental cells, they were incubated for 10 minutes at 25° C. before the first reading was taken. Thereafter readings were taken at 340 millimicrons at 2- to 4-minute intervals for 12 to 20 minutes. No endogenous activity was subtracted from the rates, since controls in several experiments showed that after the initial incubation the A_{340} of homogenate plus cytochrome without added DPNH remained constant. Because of the high absorption of reduced DPN, the use of blank cuvettes without substrate was not feasible.

Succinic dehydrogenase. Spectrophotometric method of Slater and Bonner (1952). The experimental cuvettes contained 0.01 *M* sodium cyanide, 10^{-3} *M* potassium ferricyanide, 0.02 *M* sodium succinate, 0.1 *M* phosphate, pH 7.4, and 0.1 to 0.4 ml. of 10% homogenate. The blank cuvette contained only homogenate in 0.1 *M* phosphate. After the addition of all components the cuvettes were incubated at 25° C. for 10 minutes before the first reading was taken. The absorbence at 410 millimicrons was measured at 5- or 10-minute intervals for 25 to 40 minutes.

Isocitric dehydrogenase. Spectrophotometric method of Ochoa (1948). The experimental cuvette contained 0.02 *M* Tris (hydroxymethyl) aminomethane, pH 7.5, 7×10^{-4} *M* isocitrate, 5×10^{-3} *M* sodium cyanide, 4.5×10^{-5} *M* triphosphopyridine nucleotide (TPN), 6×10^{-4} *M* $MnCl_2$, and 0.1 or 0.2 ml. of 10% homogenate. The blank contained all components except isocitrate. Readings at 340 millimicrons were begun immediately after the addition of homogenate to both blank and experimental cells, and they were continued at 2- or 3-minute intervals for 8–10 minutes.

Alpha-ketoglutaric dehydrogenase. Method of Sanadi and Littlefield (1951). Experimental cuvettes contained 0.1 *M* phosphate, pH 7.7, 2.5×10^{-3} *M* cocarboxylase, 5×10^{-4} *M* sodium cyanide, 8.6×10^{-5} *M* 2,6-dichlorophenolindophenol, 3.5×10^{-3} *M* magnesium chloride, 6.7×10^{-3} *M* alpha-ketoglutarate, pH 7.7, and 0.1 or 0.2 ml. of 10% homogenate. The blank contained all components except substrate. The readings were begun immediately after the addition of homogenate, and 4 readings at 600 millimicrons were taken at 2-minute intervals. In one experiment ferricyanide was used as the hydrogen acceptor in the assay of this enzyme by the method of Stumpf *et al.* (1947). The cuvettes contained similar amounts of all components, except that 10^{-3} *M* potassium ferricyanide was substituted for the 2,6-dichlorophenolindophenol, and the decrease in optical density was read at 410 millimicrons for 25–30 minutes.

Fumarase. Spectrophotometric method of Racker (1950). Experimental cuvettes contained 0.05 *M* malate in 0.05 *M* phosphate, pH 7.4; blank cuvette contained only phosphate. After addition of 0.05 or 0.1 ml. of 10% homogenate to both blank and experimental vessels, the absorbence at 240 millimicrons was measured at 2-minute intervals for 10 minutes.

Malic dehydrogenase. Method of Mehler *et al.* (1948). The experimental cuvette contained 0.02 *M* Tris buffer, pH 7.5, 10^{-3} *M* sodium cyanide, 5.1×10^{-5} *M* oxalacetate, pH 7.5, 1.7×10^{-4} *M* DPNH, and 0.05 to 0.1 ml. of 1% homogenate. The substrate was added last, and readings were taken at 340 millimicrons at 15-second intervals for one minute. The oxalacetate solution was prepared just before use and kept on ice. Not more than 10 minutes elapsed between the preparation of this substrate and the enzyme assay. The amounts of pyruvic acid formed by spontaneous decarboxylation of the oxalacetate were considerd to be negligible. At least four determinations of enzyme activity were always made.

Aconitase. Method of Racker (1950). The experimental cuvettes contained 0.03 *M* citrate in 0.05 *M* phosphate, pH 7.4, and 0.05 or 0.1 ml. of 10% homogenate. The blank cuvette contained only homogenate and buffer. The increase in absorbence at 240 millimicrons was determined at 2-minute intervals between 5 and 15 minutes after the start of the reaction.

The following values (in cm.^{2}/mole $\times 10^6$) were used for the molar extinction coefficients of the substances used in the assays: DPNH and TPNH, 6.22 at 340 millimicrons (Horecker and Kornberg, 1948), cis-aconitate, 3.30 at 240 millimicrons (Racker, 1950), ferricyanide, 1.00 at 410 millimicrons (Strittmatter and Velick, 1956), and 2,6-dichlorophenolindophenol, 18.5 at 600 millimicrons (Sanadi and Littlefield, 1951).

The sources of materials used in the assays were: Tris (hydroxymethyl) aminomethane, EDTA, citric acid, succinic acid, and ascorbic acid, Will Corporation; potassium ferricyanide and 2,6-dichlorophenolindophenol, Fisher Chemical Company; DPN (95–100%), DPNH (Type I, 90–95%), and thiamine pyrophosphate (cocarboxylase, 80–90%), Sigma Chemical Company; and cytochrome *c* (0.34% iron), trisodium isocitrate, alpha-ketoglutaric acid, and oxalacetic acid, Nutritional Biochemicals Corporation.

Stock solutions of coenzymes and substrates used in the assays were made and frozen in several small batches, so that only one or two thawings were necessary in using each batch. Stock solutions of other chemicals were stored at 2–4° C. Before beginning each experiment, sufficient quantities of all solutions were made so that, with the exception of the assay for malic dehydrogenase, all determinations during any 2-day experiment were made from the same stock solutions. The concentrations of the pyridine nucleotide coenzymes were determined at intervals during some 2-day experiments to ascertain whether decomposition of these materials had occurred during storage.

RESULTS

Respiration. Cleland (1950) has reported that in the eggs of *Ostrea* respiration rises at least until the blastula stage. To this author's knowledge, no other investigation of respiratory changes during early development of the oyster has been made. The results of the present series of respiration measurements are presented in Table I. The rate of respiration of the blastula was found to be about three times that of the egg at the first cleavage. A further three-fold increase in rate occurred between the blastula stage (9 hours) and the trochophore stage (24 hours). The respiratory rate of the two-day-old larva (early veliger) was not found to be significantly different from that of the trochophore. The levelling-off of respiratory rate at the trochophore stage is most likely not due to starvation, since oyster embryos cultured at 20° apparently do not begin feeding until they are about 60 hours old (Amemiya, 1926).

TABLE I

Rates of respiration of developing eggs of Crassostrea virginica. *The values represent micromoles of oxygen taken up per minute by one million embryos. Numbers in parentheses in headings refer to hours after fertilization. Embryos were grown at 20° C., and respiration was measured manometrically at 25° C. for periods of about one hour*

Expt.	Cleaving eggs (1-1½ hrs.)	Blastulae (8–10 hrs.)	Trochophores (23–25 hrs.)	Early veligers (48–50 hrs.)
1	0.027	0.076	0.180	
2	0.024	0.045	0.153	
3	0.021		0.219	
4	0.020	0.109	0.162	
5			0.159	0.139
6			0.215	0.142
7			0.182	0.179
8			0.284	0.234
9			0.203	0.181
10	0.029	0.067		0.100
Average	0.024 ±0.003	0.074 ±0.023	0.195 ±0.039	0.163 ±0.042

Succinic dehydrogenase, DPNH-oxidase, and cytochrome oxidase. In measuring the activities of these enzyme systems it was desirable to determine the conditions under which maximal rates of electron transfer could occur in homogenates. In preliminary experiments the effects of pH, buffer concentration, and substrate concentration on the activities of the succinic dehydrogenase and DPNH-oxidase were tested. Variation of pH between 7.2 and 7.8, of succinate between 0.01 and 0.04 *M*, of phosphate between 0.05 and 0.15 *M*, and of ferricyanide between 10^{-3} and 2×10^{-3} *M* had but little effect on the activity of succinic dehydrogenase. In one experiment the rate of reduction of cytochrome *c* by a homogenate of fertilized eggs was compared with the rate of ferricyanide reduction, when the homogenate was oxidizing succinate. The rate of transfer of electrons to cytochrome *c* was measured by determining manometrically the activity of the succinoxidase system, in which succinic dehydrogenase is the rate-limiting factor, when cytochrome *c* is added to the system. This determination, made by the method of Schneider and Potter (1943), indicated that the cytochrome was reduced at a rate about one-third that of the ferricyanide (measured by the spectrophotometric method). This result is similar to that of Green *et al.* (1955), who found that highly purified preparations of the succinic dehydrogenase complex from beef-heart mitochondria reduced

TABLE II

Activities of succinic dehydrogenase, DPNH-oxidase, and cytochrome oxidase in homogenates of embryos of the oyster. Homogenates were made in 0.03 M phosphate, pH 7.4. Assays were performed at 25° C. See text for details of assay systems. Values represent micromoles of substrate (ferricyanide, DPNH, or oxygen) utilized per minute by one million embryos

Expt.	Cleaving egg	Blastula	Trochophore	Veliger
Succinic dehydrogenase				
1	0.190	0.160	0.135	
2	0.155	0.090	0.155	
3	0.170		0.175	
4	0.110	0.185	0.175	
6			0.185	0.155
7			0.105	0.130
8			0.255	0.135
9			0.135	0.145
10	0.098	0.126		0.085
Average	0.145	0.140	0.164	0.130
	±0.035	±0.036	±0.042	±0.024
DPNH-oxidase				
1	0.111	0.098	0.116	
2	0.131	0.088	0.077	
3	0.118		0.121	
4	0.081	0.130	0.097	
6			0.129	0.083
7			0.134	0.139
8			0.210	0.125
9			0.167	0.085
10	0.115	0.074		0.078
Average	0.111	0.098	0.144	0.102
	±0.017	±0.026	±0.035	±0.025
Cytochrome oxidase				
1	0.383	0.445	0.368	
2	0.408	0.389	0.578	
3	0.362		0.339	
4	0.362	0.618	0.492	
6			0.337	——*
7			0.300	0.177
8			——*	0.215
9			0.535	0.284
10	0.393	0.325		0.330
Average	0.382	0.444	0.421	0.252
	±0.018	±0.106	±0.122	±0.059

* Activity not proportional to concentration of homogenate.

ferricyanide more rapidly than cytochrome *c* and also more rapidly than several other artificial electron-acceptors.

In preliminary experiments with DPNH-oxidase, it was found that this enzyme system was inhibited about 30% by a 2-fold excess of DPNH or by a 2-fold excess of cytochrome *c*. These effects have also been reported for the DPNH-oxidase system of chick embryos by Brand and Mahler (1959). The optimum pH for this system was found to be 7.4. The activity of the system was decreased in phosphate concentrations below 0.05 *M*, and occasionally was found to be somewhat higher in 0.1 *M* than in 0.05 *M* buffer; however, the latter effect was not consistently obtained. In several experiments the activity of the DPNH oxidase was shown to be inhibited 95% or more by 10^{-3} *M* cyanide. In the absence of added cytochrome, the DPNH oxidase activity was very low in most experiments.

Since the activity of cytochrome oxidase was always greater than the combined activities of the two systems above, no special effort was made to determine the conditions for maximal activity of this enzyme. In preliminary experiments it was found that neither the concentration of cytochrome *c* nor that of ascorbic acid was a limiting factor in the assays. The effects of variation of pH or buffer concentration were not tested.

The results of measurements of these components of the electron transport system are presented in Table II. Since relatively large amounts of homogenate were required for the assays, the experiments were divided into two series. In the first, measurements were made betwen the first cleavage and 24 hours; in the second the rates of enzyme activity in 24- and 48-hour stages were compared. No consistent change was observed in the activity of succinic dehydrogenase or of DPNH oxidase during development to the veliger. (The average value for DPNH oxidase at 24 hours is 30–40% higher than those in the blastula and veliger; this difference is considered to be too small to be significant in view of the variation between values obtained at each stage in different experiments.)

Cytochrome oxidase is also nearly constant in activity up to the trochophore stage. A considerable decrease in the activity of this enzyme between 24 and 48 hours was found in most experiments; moreover, in one measurement at each of these stages the activity was not proportional to the concentration of homogenate. These findings may indicate the presence of an inhibitor of this enzyme in late stages, as suggested by Deutsch and Gustafson (1952) for homogenates of sea urchin blastulae. A slight clumping of homogenates of trochophores and veligers usually occurred in the presence of 2×10^{-4} *M* cytochrome *c*. This did not appear to affect the relationship between concentration of homogenate and enzyme activity in most manometric experiments. In lesser concentrations, such as those used in the assay of DPNH oxidase, no clumping of the homogenates occurred.

A comparison of respiratory rates during development (Table I) with the enzyme activities reported in Table II shows that the terminal enzyme systems must transfer electrons at an increasing rate as development progresses in order to account for the increase in respiration. Such an increased rate of transfer could be due to the greater rate of production of succinate and reduced pyridine nucleotides, or it could be a result of the synthesis of some rate-limiting component of the terminal system, such as cytochrome *c*. Cytochrome *c* was not a limiting factor in any of the assay-systems; it is therefore not possible from present data to determine whether it is rate-limiting for the respiration at any stage of development. Data presented in the next section indicate that at least two of the enzymes of the citric acid cycle do increase in activity during one phase of development.

Isocitric dehydrogenase and alpha-ketoglutaric dehydrogenase. The levels of activity of these enzymes at each embryonic stage investigated are listed in Table III. In contrast to the terminal enzymes, a marked increase in the activity of isocitric dehydrogenase was observed between 9 and 24 hours in all experiments. Thus in the trochophore the level of this enzyme is about three times the level in the blastula. This increase is about equivalent to the increase in respiration during the same period. In no case was any increase in this enzyme observed prior to the blastula stage. In some experiments, decreases in the activity of isocitric dehydrogenase were found between 24 and 48 hours. The activity of this enzyme was nearly 100% higher in sucrose homogenates than in homogenates made in 0.03 *M* Tris. The addition of sucrose to homogenates made in dilute buffer also enhanced the activity by as much as 50%. The reason for this effect is not known.

TABLE III

Activities of isocitric and alpha-ketoglutaric dehydrogenases in homogenates of oyster embryos. Homogenates were made in 0.3 M sucrose and 0.05 M Tris (hydroxymethyl) aminomethane, pH 7.4. Assays were performed at 25° C. (for details see text). Values represent micromoles of substrate utilized per million embryos per minute

Expt.	Cleaving egg	Blastula	Trochophore	Veliger
		Isocitric dehydrogenase		
11	0.080	0.070	0.330	0.100
12	0.069		0.210	0.150
13	0.050	0.043	0.190	0.140
14	0.104	0.131	0.146	0.289
15	0.083	0.088	0.284	0.277
16	0.098	0.109	0.272	0.256
Average	0.097 ± 0.024	0.088 ± 0.030	0.239 ± 0.060	0.202 ± 0.074
		Alpha-ketoglutaric dehydrogenase		
11*	0.084*		0.220*	0.290*
13	0.024	0.018	0.055	0.033
14	0.021	0.021	0.038	0.038
15	0.028	0.020	0.050	0.058
Average**	0.024 ± 0.003	0.020 ± 0.001	0.048 ± 0.007	0.043 ± 0.011

* Assayed with ferricyanide.
** Data for 2,6 dichlorophenolindophenol only.

The levels of activity of alpha-ketoglutaric dehydrogenase, shown in Table IV, indicate that approximately a 2.5-fold increase in the activity of this enzyme occurs between 9 and 24 hours of development. As in the case of isocitric dehydrogenase, little change in the activity of this enzyme occurs during cleavage or during development from trochophore to veliger. In one experiment the rate of reduction of ferricyanide was used as a measure of the activity of this enzyme. This hydrogen acceptor was reduced at a rate which was 5–10 times higher than that of the 2,6-dichlorophenolindophenol; however, the high molar extinction of the latter made it more desirable for assays of homogenates with low activities. The assays were complicated by the high rates of endogenous reduction of this dye, and by the rapid loss of enzyme activity with either acceptor. In most homogenates prepared in dilute Tris or phosphate buffer, the activity of alpha-ketoglutaric dehydrogenase was too low to measure. Data are therefore presented only for homogenates made in buffered sucrose.

Table IV

Activities of malic dehydrogenase and aconitase in homogenates of oyster embryos. Homogenates were made in 0.03 M Tris buffer, pH 7.4. Assays were performed at 25° C. Values represent micromoles of substrate utilized per minute by one million embryos

Expt.	Cleaving egg	Blastula	Trochophore	Veliger
Malic dehydrogenase				
17	9.2	9.3	15.6	10.0
18	10.0		14.5	12.0
19	8.5	10.5	10.3	12.6
20	7.0	8.2	7.7	8.0
21	8.0	9.1	7.2	9.3
22	8.5	8.1	7.0	6.0
Average	8.5 ± 0.9	9.2 ± 0.8	10.4 ± 3.5	9.7 ± 2.2
Aconitase				
17	0.068	0.082	0.123	0.106
18	0.082	0.080	0.110	0.090
19	0.080	0.088	0.120	0.090
20	0.095	0.110	0.063	0.104
21	0.100	0.110	0.065	0.070
Average	0.085 ± 0.011	0.094 ± 0.013	0.096 ± 0.026	0.093 ± 0.013

Fumarase, malic dehydrogenase and aconitase. Efforts to measure fumarase in whole homogenates, prepared in either sucrose or in dilute buffer, were unsuccessful. Although the enzyme was found to be present in all stages by the spectrophotometric method, its activity was extremely low and was not proportional to the homogenate concentration in most cases. In contrast to fumarase, the activity of malic dehydrogenase is extremely high in all stages of development, being at least 20 times that of any other enzyme measured (Table IV). High activities have been noted for this enzyme in other animal tissues (*cf.* Krebs and Lowenstein, 1960). Little change in activity was found during development to the veliger stage.

The presence of aconitase in all stages of development is of interest, since this enzyme has been reported to be absent from adult oyster-mantle (Jodrey and Wilbur, 1955). As in the case of malic dehydrogenase, no change in activity occurs during any phase of early development (Table IV). Neither the activity of aconitase nor that of malic dehydrogenase was affected by the composition of the medium used for homogenization; the data are therefore given for homogenates made in 0.03 *M* Tris buffer.

DISCUSSION

The data reported above indicate that: (1) although respiration increases 3-fold during cleavage, little change in activity of any of the enzymes of the citric acid cycle occurs before the blastula stage is reached; (2) a further 3-fold increase in respiration between blastula and trochophore stages is paralleled by increases in two enzymes of the Krebs cycle; and (3) between trochophore and veliger stages there is little change in respiration or in enzyme activity, with the possible exception of a decrease in cytochrome oxidase. With regard to the period of cleavage, the results are similar to those of Gustafson and Hasselberg (1951) for the sea urchin and to those of Spiegelman and Steinbach (1945) and Petrucci (1957) for two amphibians. Following the blastula stage of the oyster marked increases were found in the activities of isocitric dehydrogenase and alpha-ketoglutaric dehydrogenase. Such increases in activity of two enzymes which are "biochemically adjacent" in the citric acid cycle are of considerable interest, and it would be desirable to determine whether the changes occur simultaneously or sequentially.

No change in activity of most enzymes of the Krebs cycle or of the electron-transport system occurs during development to the veliger stage in the oyster. This finding is in contrast to that of Gustafson and Hasselberg (1951) for the sea urchin, in which both succinic dehydrogenase and malic dehydrogenase increase 4- to 5-fold between blastula and pluteus stages. These authors have postulated that an increase in the number of mitochondria is responsible for changes in activity of these and other enzymes during this period of development. Counts of granules exhibiting the staining properties of mitochondria in developing sea urchins have given supporting evidence for this hypothesis, since the number of these granules apparently increases during about the same period of development (Gustafson and Lenicque, 1952; Shaver, 1956).

In the unfertilized egg of the oyster Cleland (1951) has obtained evidence for the localization of succinic oxidase and cytochrome oxidase in cytoplasmic granules. and he has shown that removal of the granules from homogenates by centrifugation drastically reduces the ability of the homogenates to take up oxygen in the presence of substrates of the Krebs cycle. This latter finding may be simply due to the removal of the terminal electron-transport systems, which would reduce the respiration of the homogenates even in the presence of substrate. Since his results clearly indicate the localization of succinic oxidase and cytochrome oxidase in the large granules, it is possible to conclude from the present study that any changes in number of granules during development of the oyster are not accompanied by changes in these terminal enzymes. Data on the concentrations of other enzymes of the citric acid cycle in the large granule fraction from homogenates of eggs and larvae are presented in an accompanying report (Black, 1962).

The author is indebted to Mr. James Egan and to Miss Lynn Search for their technical assistance during this investigation. A preliminary report of these results has been previously published (Black, 1960).

SUMMARY

1. Measurements of respiration, cytochrome oxidase, and the DPNH oxidase system, as well as five enzymes of the citric acid cycle, aconitase, isocitric dehydrogenase, alpha-ketoglutaric dehydrogenase, succinic dehydrogenase, and malic dehydrogenase, have been made in oyster embryos between the first cleavage and the early veliger stage. The rate of respiration increases 9-fold to the trochophore stage and levels off until the veliger stage is reached. Succinic dehydrogenase, DPNH oxidase, malic dehydrogenase and aconitase were not found to change appreciably during development to the veliger. Cytochrome oxidase showed no significant change prior to the trochophore stage, but decreases were found in this enzyme after this stage. Isocitric dehydrogenase and alpha-ketoglutaric dehydrogenase were found to increase, paralleling the increase in respiration, between the blastula and trochophore stages. Following this stage, these enzymes remain constant in activity up to the veliger stage.

2. The results are shown to be in contrast to those obtained for the sea urchin by other workers. The possible relationships between changes in enzyme activities and increases in respiratory rate are considered.

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THE CONCENTRATIONS OF SOME ENZYMES OF THE CITRIC ACID CYCLE AND ELECTRON TRANSPORT SYSTEM IN THE LARGE GRANULE FRACTION OF EGGS AND TROCHOPHORES OF THE OYSTER, CRASSOSTREA VIRGINICA[1]

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In an accompanying report Black (1962) has shown that considerable changes in the relative activities of several enzymes of the tricarboxylic acid cycle occur during early development of the oyster. Thus, between the blastula and trochophore stages two enzymes, TPN-specific isocitric dehydrogenase and alpha-ketoglutaric dehydrogenase, increase 2- to 3-fold, roughly paralleling the increase in respiration during this same period. Five other enzymes were found not to change appreciably during development to the trochophore. These were: aconitase, succinic dehydrogenase, malic dehydrogenase, DPNH oxidase, and cytochrome oxidase. Following the trochophore stage cytochrome oxidase decreased somewhat in activity, while the other enzymes remained constant.

With the exception of isocitric dehydrogenase, most of the enzymes studied are known to be present in considerable concentration (though not usually localized) in the mitochondrial fraction of vertebrate tissues. The relationship of enzymic changes to the possible biochemical differentiation of these respiratory granules must therefore be considered. Previous investigations pertaining to this phenomenon include the finding that the content of cytochrome oxidase increases in mitochondria of differentiating rat muscle (Shen, 1955), the work reported by Weber and Boell (1955) and Boell and Weber (1955) in which an increase in the content of cytochrome oxidase and succinoxidase in mitochondria of *Xenopus laevis* was found during development, and the investigation of Mahler, Wittenberger and Brand (1958) in which changes in the relative activities of several respiratory enzymes were found to occur in the large granule fraction of homogenates of the chick between 2 and 6½ days of development.

In eggs of marine invertebrates few studies have been made of the distribution of respiratory enzymes in the various cell fractions which can be obtained from homogenates. In the sea urchin mitochondria isolated in sucrose from unfertilized eggs have been shown to contain succinoxidase and cytochrome oxidase (Maggio and Ghiretti-Magaldi, 1958). The latter enzyme undergoes a 30% increase in the mitochondria at fertilization, but shows no further change until the blastula stage is reached (Maggio, 1959). Cytochemical studies of eggs of various invertebrates, in which respiratory enzymes (usually indophenol oxidase and succinic dehy-

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drogenase) have been reported to be associated with granules, are reviewed by Brachet (1960). In eggs of the oyster Cleland (1951) has studied oxygen uptake by homogenates in the presence of substrates of the citric acid cycle, and has found that removal of the large granules by centrifugation results in a decreased ability of the homogenates to respire in the presence of such intermediates. In addition, he has shown that succinoxidase and cytochrome oxidase are localized in the large granules.

In the present study an attempt has been made to determine the distribution of several enzymes involved in aerobic respiration between the "large-granule" fraction (yolk, mitochondria and other granules) and the "supernatant" fraction (submicroscopic and soluble elements) derived from sucrose homogenates of fertilized eggs and trochophores of the oyster, *Crassostrea virginica*. Several changes in enzyme distribution and in enzyme content of the large granules will be shown to occur during this developmental period.

Materials and Methods

The oysters used in this study were collected by dredging from the Rappahannock River and stored in trays at Gloucester Point, Virginia. The eggs were removed from gonads, inseminated, and cultured by methods described previously (Black, 1962). The stages used for homogenization were cleaving eggs (1½ hours after fertilization) and late trochophores (20 hours after fertilization when cultured at 22° C.). The eggs and larvae were collected by centrifugation and homogenized in 10–15 volumes of 0.55 *M* sucrose buffered with 0.05 *M* Tris (hydroxymethyl) aminomethane, pH 7.35. This medium is approximately isotonic to the dilute sea water in which eggs were grown. The addition of as much as 5% polyvinyl pyrrolidone to the medium caused considerable clumping of the granules in homogenates; this component was therefore omitted from the homogenization mixtures. Homogenization of cleaving eggs was performed by forcing the suspension through a 22-gauge needle; usually 3–5 minutes were required for complete disruption of the eggs. Partial homogenization of trochophores required 15–20 minutes of this treatment, or two minutes of blending in a Lourdes multimixer at 16,000 rpm. No attempt to achieve complete homogenization of the trochophores was made. The homogenization and all subsequent operations were carried out at 0° C.

The homogenates were divided into two aliquots of 2 ml. each, and each aliquot was centrifuged for 10 minutes at 1,000 × gravity in a Lourdes Model LR refrigerated centrifuge containing a swinging bucket rotor. The sediment from the first centrifugation was washed once with 2 ml. of buffered sucrose, and the two supernatant fractions were combined in each of the two aliquots. The washed, low-speed sediment contained some nuclei but consisted chiefly of whole cells and embryonic coats, and it was discarded from both aliquots.

Both aliquots of the homogenate, minus nuclei, whole cells, and embryonic coats, were centrifuged at 18,000 × gravity for one hour in order to sediment the granules. This centrifugal force was near the maximum which could safely be obtained with the rotor available. In preliminary experiments a centrifugation time of one hour was found to be barely sufficient to sediment nearly all of the visible granules as well as all of the succinic dehydrogenase and DPNH oxidase from homogenates of fertilized eggs. In the preliminary experiments, the supernatant fluid recovered from this high-speed centrifugation of egg homogenates was centrifuged for an additional hour at the same force. This second treatment failed to cause the sedimentation of a usable quantity of granules.

The high-speed sediment in one of the aliquots of homogenate was resuspended in 4 ml. of buffered sucrose and washed by centrifugation for an additional hour. The other aliquot served as a control, in which the sediment was merely resuspended in the original supernatant fluid. This aliquot, labelled "whole homogenate" (minus nuclei), was centrifuged again during the washing of the granules. Supernatant fractions from the first and second centrifugal treatments of the "experimental" aliquot were combined, and the volume was noted. The washed granules were diluted to 4.0 ml. in buffered sucrose. Enzyme determinations were then made on the washed granules, the combined supernatant fractions, and the "whole homogenate" (combined granule and supernatant fractions). In addition, the fatty fraction found at the top of the first supernatant portion of the fertilized eggs was removed with a spatula, suspended in 1 ml. of buffered sucrose, and assayed for all enzymes. Since no enzyme was found to be concentrated in this fraction, the assays were not repeated on the fat from homogenates of trochophores.

The enzymes measured in the separate fractions were: aconitase, isocitric dehydrogenase, alpha-ketoglutaric dehydrogenase, succinic dehydrogenase, fumarase, malic dehydrogenase, DPNH oxidase (with and without added cytochrome *c*), and DPNH-cytochrome *c* reductase. Simultaneous assays of each enzyme were usually conducted on corresponding fractions from three separate batches of eggs or embryos. Each experiment was repeated once, so that all of the reported values represent the averages of data which were obtained from at least six batches of eggs or embryos. Usually not more than one or two enzymes could be measured in any one experiment since it was desirable to complete the determinations within 1 or 1½ hours after preparation of the fractions. Succinic dehydrogenase was measured in all of the granule preparations for use as a reference enzyme.

Spectrophotometric methods used in assaying all of the above enzymes except DPNH-cytochrome *c* reductase have been listed previously (Black, 1962). The latter enzyme was determined by the method of Strittmatter and Velick (1956). The cuvettes contained 10^{-4} M cytochrome *c,* 10^{-3} M sodium cyanide, 1.7×10^{-4} M DPNH, 0.05 M phosphate, pH 7.4, and 0.1 or 0.2 ml. of homogenate or homogenate-fraction in a total volume of 3.0 ml. The changes in A_{550} were followed at 15-second intervals for one or two minutes after the addition of enzyme. Assays of all of the enzymes were performed at 25° ± 1° C.

RESULTS

Microscopic examination of homogenate fractions. No serious effort was made to characterize any component of the large-granule fraction. Under oil phase the granules were seen to consist of both spherical and rod-shaped bodies of a wide range of sizes. Attempts to stain with Janus green were partially successful, as determined on masses of granules which were collected by centrifugation after staining; however, it was difficult to observe staining of individual granules by use of the microscope. The supernatant fraction was not found to possess visible granules under ordinary lighting or phase; however, under dark-field illumination small light-scattering particles could be seen.

Enzyme distributions in granule and supernatant fractions. The percentages of total enzyme activities recovered in the large granules of eggs are listed in Table I, column 2. These percentages are based on the sums of the activities recovered in both fractions. In the egg, fumarase, malic dehydrogenase, and DPNH-cytochrome *c* reductase are found almost entirely in the supernatant fluid, whereas appreciable percentages of all of the other enzymes are found in the granules. The electron-transport enzymes, succinic dehydrogenase and DPNH oxidase, are recovered almost exclusively in the granules, as might be expected if this fraction contains nearly all of the mitochondria. The finding that an active DPNH-cytochrome *c* reductase is almost entirely localized in the supernatant fraction from egg homogenates is of interest, since this enzyme has been reported to be present in high concentration in the microsomal fraction of mammalian liver (see Strittmatter and Velick, 1956). None of the enzymes listed were found to be present in quantity in the fatty fraction of the egg.

TABLE I

Distribution and total recovery of respiratory enzymes in homogenate-fractions of fertilized eggs and trochophores. Homogenates in 0.55 M sucrose containing 0.05 M Tris, pH 7.35, were freed of nuclei and centrifuged at 18,000 × gravity for one hour. The sediment was washed once by the same treatment and the two supernatant fractions were combined. Whole homogenates were also freed of nuclei and whole cells for comparison with above fractions. Standard deviations are based on six determinations, in separate batches of embryos, of each value

Enzyme	Percentage of total recovered enzyme present in granules based on 100% for the sum of the activities in granules and supernatant fraction		Percentage recovery of enzyme in granules plus supernatant fluid based on 100% for the whole homogenate	
	Fertilized egg	Trochophore	Fertilized egg	Trochophore
Aconitase	37.5 ± 9.1	25.7 ± 5.0	88.7 ± 8.1	108.6 ± 27.3
Isocitric dehydrogenase	65.1 ± 4.9	63.3 ± 1.6	80.5 ± 12.8	91.3 ± 18.0
Alpha-ketoglutaric dehydrogenase	63.9 ± 6.7	100.0 ± 0.0		
Succinic dehydrogenase	100.0 ± 0.0	67.1 ± 2.0	77.6 ± 13.4	98.9 ± 8.9
Fumarase	3.0 ± 1.3	16.1 ± 3.5	302.8 ± 128.6	135.9 ± 15.0
Malic dehydrogenase	8.5 ± 2.4	11.2 ± 2.2	89.5 ± 9.1	112.6 ± 11.7
DPNH oxidase (without cytochrome *c*)	92.0 ± 1.6	46.9 ± 5.8	84.0 ± 13.2	87.3 ± 15.6
DPNH oxidase (with added cytochrome *c*)	92.1 ± 2.9	60.1 ± 5.3	84.7 ± 6.1	81.9 ± 10.6
DPNH-cytochrome *c* reductase	6.3 ± 1.8	23.9 ± 4.8	123.7 ± 14.5	94.5 ± 15.7

The average per cent recovery of each enzyme in the granule fraction of trochophores is given in Table I, column 3. Major increases in the percentages of alpha-ketoglutaric dehydrogenase, fumarase, and DPNH-cytochrome *c* reductase recovered in the granules are found when these data are compared with those which were obtained for egg homogenates. Alpha-ketoglutaric dehydrogenase appears to be localized in the granules of trochophores, and the percentage of fumarase and

DPNH-cytochrome *c* reductase in the trochophore granules are 4 to 5 times higher than in the granules obtained from eggs. In contrast to these enzymes, succinic dehydrogenase and DPNH oxidase in the trochophore homogenates were found to be distributed between the granule and the supernatant fractions, so that about $\frac{1}{3}$ of the total recovered enzyme was found in the supernatant fluid in each case. Centrifugation of the trochophore homogenates for 2 hours at 18,000 × gravity did not result in increased sedimentation of either enzyme.

In Table 1, columns 4 and 5, the sums of the recovered enzyme activities in the separate fractions are expressed as percentages of the activities found in the "whole homogenates" (minus nuclei). With the exceptions of fumarase and DPNH-cytochrome *c* reductase, the recoveries of most enzymes are somewhat higher in the trochophore fractions than in those of fertilized eggs. This difference may be an indication that there are substances in whole homogenates of trochophores which inhibit enzyme activities, or that the enzymes in the separated fractions from trochophores are somewhat more stable than in those from eggs. Because of the high endogenous activity of whole homogenates with the dye, 2, 6-dichlorophenolindophenol, the activities of alpha-ketoglutaric dehydrogenase were not measured in the whole homogenates. Endogenous reduction of this dye was almost negligible in the separated fractions.

The extremely high recovery of fumarase in separated fractions of eggs and trochophore homogenates is of particular interest. As noted previously (Black, 1962), fumarase activity is extremely variable in whole homogenates of all stages. In the separated fractions of eggs, nearly all of the activity was present in the supernatant fluid; the inhibition in whole homogenates therefore appears to be caused by the presence of the granules. Since the inhibition is obtained when either fumarate or malate is used as the substrate, it does not appear to be a result of any competing reaction which might be catalyzed by the granules. A marked reduction in the total recovery from separate fractions is observed in the trochophore; this may indicate that trochophore granules inhibit the enzyme to a lesser extent than granules from eggs. A calculation of the total fumarase activity in eggs has been made from the data obtained on the separate fractions. This calculation shows that one million eggs have sufficient enzyme to convert 0.442 ± 0.098 micromoles of malate to fumarate per minute. The ratio of total fumarase to total succinic dehydrogenase in the trochophore is not appreciably different from that in the egg; fumarase activity therefore probably does not change during this period of development.

An excessive recovery of DPNH-cytochrome *c* reductase is also found in separated fractions of the egg, but not in those of the trochophore (Table I). A slight inhibition of this enzyme by the granules of the egg again appears to be responsible for the high recovery. The total activity of this enzyme in all fractions of the egg is calculated to be sufficient to reduce 0.448 ± 0.056 micromoles of cytochrome *c* per minute per million eggs, and the ratio of total reductase to total DPNH oxidase is the same in eggs and trochophores.

Ratios of enzyme activities in granules. The findings summarized in Table I, together with the data available from assays of enzymes in whole homogenates (Black, 1962), indicate that changes in the relative activities of respiratory enzymes in the large granules must occur during development. In order to determine more precisely the extent of the changes in the granules, measurements of the ratios of the activities of these enzymes to that of succinic dehydrogenase have been made on the granule fractions prepared from eggs and from trochophores. The average ratios obtained are presented in Table II. The averages have been calculated from data obtained in 6 to 10 separate determinations of each ratio. The only enzyme which was found to be constant in the two stages in comparison to the reference enzyme was DPNH oxidase in the presence of added cytochrome *c*. This enzyme system had an activity which was almost exactly equal to that of succinic dehydrogenase in the granules of both eggs and trochophores (ferricyanide was used as the electron acceptor in all assays of the reference enzyme). In the absence of added cytochrome the activity of DPNH oxidase in the egg granules was about ½ of the maximum activity. A decrease of 29% in the activity of the unsupplemented DPNH oxidase system, relative to that of the reference enzyme, was found in the trochophore granules; presumably a loss of endogenous cytochrome *c* from the granules was responsible for this change.

Table II

Ratios of activities of respiratory enzymes to that of succinic dehydrogenase in granules of fertilized eggs and trochophores. Ratios are expressed as micromoles of substrate utilized per minute divided by micromoles of ferricyanide reduced per minute by succinic dehydrogenase. All ratios were determined at 25° C. Standard deviations are based on 10 determinations of each ratio for isocitric dehydrogenase and 6 determinations of all other ratios

Enzyme	Fertilized egg	Trochophore	Per cent change in ratio	't'
Aconitase	0.431 ± 0.160	0.606 ± 0.155	+41	2.33*
Isocitric dehydrogenase	1.350 ± 0.370	2.530 ± 0.460	+88	7.00**
Alpha-ketoglutaric dehydrogenase	0.083 ± 0.013	0.170 ± 0.050	+105	3.78*
Succinic dehydrogenase	1.0	1.0		
Fumarase	0.196 ± 0.138	0.930 ± 0.430	+382	3.61*
Malic dehydrogenase	8.16 ± 3.30	23.50 ± 5.90	+188	5.01**
DPNH oxidase (without added cytochrome *c*)	0.524 ± 0.088	0.370 ± 0.077	−29	9.06**
DPNH oxidase (with added cytochrome *c*)	0.990 ± 0.080	1.020 ± 0.240	+3	0.08
DPNH-cytochrome *c* reductase	0.343 ± 0.130	1.560 ± 0.350	+355	14.31**

*$P < 0.005$

** $P < 0.001$

With the exception of the above enzyme system, all of the enzymes investigated were found to have considerably higher activities, relative to that of succinic dehydrogenase, in the trochophore granules than in those of fertilized eggs. The percentage increases in ratio range from 41 for aconitase to 382 for fumarase (Table II). An analysis of the data given in Table II shows that the probability is less than 0.005 that the differences found between the egg and trochophore granules for all enzymes except cytochrome-supplemented DPNH oxidase are due to random variations in ratios.

Discussion

The differences in enzyme ratios in the granules between the two stages investigated are undoubtedly related to changes in distribution and in total amounts of the enzymes. These may be summarized as follows: (1) about ⅓ of the succinic dehydrogenase and DPNH oxidase are present in the supernatant fraction of the trochophores, whereas these enzymes are almost entirely localized in the granules of eggs (Table I); (2) in contrast to these enzymes, higher proportions of alpha-ketoglutaric dehydrogenase, fumarase, malic dehydrogenase, and DPNH-cytochrome *c* reductase are associated with the granules of trochophores than with the granules of eggs (Table I); and (3) a 170% increase in the total activity of isocitric dehydrogenase and a 140% increase in that of alpha-ketoglutaric dehydrogenase are found in whole homogenates during development (Black, 1962). The distribution of isocitric dehydrogenase between the two fractions is the same in eggs and trochophores, about 63–65% of this enzyme being present in the granules. The increase in relative activity of this enzyme in the granules should therefore be at least 170%. In the case of alpha-ketoglutaric dehydrogenase a change in distribution apparently occurs during development, so that 100% of the activity is recovered in the trochophore granules, whereas only 64% is present in the egg granules. The percentage increase in the granules should therefore amount to (140 × 100/64) or at least 210%. The changes actually found in these two enzymes are only about half as great as the predicted changes (Table II). One possible explanation for these discrepancies is that selective destruction of some enzymes might occur in one of the homogenate-fractions of either developmental stage, giving erroneous values for distribution of the enzymes or for the ratios of activities in the granules. This possibility seems especially applicable to alpha-ketoglutaric dehydrogenase, since the activity of this enzyme was always found to decline very rapidly during the assays.

In evaluating the data given in Tables I and II it is necessary to consider possible artifacts other than the one given above. The presence of succinic dehydrogenase and DPNH oxidase in the supernatant fraction of the trochophores could have been caused by disruption of mitochondria during homogenization; however, such disruption of mammalian mitochondria usually results in the solubilization of many of the enzymes of the citric acid cycle (see Hogeboom, 1954). In the trochophore aconitase is slightly less concentrated in the granules than in the egg, and some loss of cytochrome *c* from the trochophore granules also appears to be probable. Except for these two enzymes and the terminal enzymes mentioned above, however, no enzyme investigated is less concentrated in the trochophore granules than in the egg granules. Since the total amounts of succinic dehydrogenase and DPNH oxidase do not change during development, there appear to be only two possible ways to account for their presence in the supernatant fraction of the trochophore. These are: (1) a specific loss (either natural or artificial) of the electron-transport enzymes from the granules of the trochophore without corresponding losses of other enzymes; and (2) natural or mechanical splitting of the granules of the trochophore in such a way that the submicroscopic fragments retain a full complement of enzymes. In the latter case enzymes of the citric acid cycle might be associated with succinic dehydrogenase and DPNH oxidase in submicroscopic particles which were not recovered in the granule-fraction of the trochophore. The first possibility seems unlikely in view of the results other workers have obtained with mammalian mitochondria—if ⅓ of the terminal enzymes are lost from the granules then one would expect that even larger proportions of the other enzymes would be lost. In any case the relative increases in the granule-fraction of all of the enzymes except aconitase are too large to be accounted for by the selective loss of the reference enzyme from the granules.

The simplest interpretation of the data given in Table II is that isocitric dehydrogenase, alpha-ketoglutaric dehydrogenase, fumarase, malic dehydrogenase, and DPNH-cytochrome *c* reductase increase in the large granules during development to the trochophore. The increases in some of the enzymes are a result of the incorporation of existing enzyme molecules into the granules. This appears to be true for fumarase and DPNH-cytochrome *c* reductase and possibly also for alpha-ketoglutaric dehydrogenase and malic dehydrogenase. The increases in other enzymes (isocitric and alpha-ketoglutaric dehydrogenases) result from the incorporation of newly-synthesized enzyme molecules into the granules or from their actual synthesis by the granules. One possible result of such changes in the granules is an increase in their capacity for catalyzing oxidations via the citric acid cycle. This may be related to the large increase in respiration which occurs during development.

It is interesting to speculate on the possibility that the populations of granules in the egg and trochophore are heterogeneous with respect to the enzyme content of individual granules. Thus the relative changes noted in Table II might represent increases in the number of large granules containing high concentrations of some enzymes (fumarase and DPNH-cytochrome *c* reductase, for example) but low concentrations of others. If such heterogeneity exists it may be expected that a diversity of aerobic metabolic pathways also exists in the embryo. In marine animals several workers have obtained cytochemical evidence for the existence of more than one kind of enzyme-containing granule in the egg; however, few of the enzymes investigated are directly involved in respiration. These findings are reviewed by Pasteels (1958).

SUMMARY

1. The enzymes aconitase, TPN-specific isocitric dehydrogenase, alpha-ketoglutaric dehydrogenase, succinic dehydrogenase, fumarase, malic dehydrogenase, DPNH oxidase, and DPNH-cytochrome *c* reductase have been assayed in two fractions, large granules and supernatant, prepared from sucrose homogenates of fertilized eggs and trochophores of the oyster, *Crassostrea virginica*. Succinic dehydrogenase and DPNH oxidase are almost completely localized in the granules of the fertilized egg, but in the trochophore about ⅓ of each enzyme is found in the supernatant fraction. High percentages of aconitase, isocitric dehydrogenase, and alpha-ketoglutaric dehydrogenase are found in the granules of both stages; the latter enzyme appears to be localized in the trochophore granules. Fumarase, malic dehydrogenase and DPNH-cytochrome *c* reductase are almost absent from granules of the egg, but considerable proportions of these enzymes are found in trochophore-granules.

2. Ratios of enzyme activities in the granules relative to that of succinic dehydrogenase have been determined at the two developmental stages. All of the enzymes except DPNH oxidase increase in activity, relative to the reference enzyme, in the granules during development to the trochophore stage. The activity of

DPNH oxidase in the presence of cytochrome *c* is about the same as that of the succinic dehydrogenase in both stages. These changes appear to indicate that differentiation of the population of respiratory granules occurs during development of the oyster.

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FEEDING MECHANISM OF THE ECHIUROID, OCHETOSTOMA ERYTHROGRAMMON LEUCKART & RUEPPELL, 1828

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The feeding mechanism of both *Urechis caupo* and *Echiurus echiurus* has been described. The former (Fisher and MacGinitie, 1928) secreted a mucus tube 2–8 inches long, the open upper end of which was fastened to the burrow near its opening, while the lower end remained attached to the body. This tube filtered the water flowing through the burrow, became loaded with food particles and was subsequently swallowed. *Echiurus echiurus* fed intermittently in aquaria (Gislén, 1940). Periods of feeding of 1–2 days alternated with rest periods lasting several days. During each feeding period food collection occurred at intervals of 20 minutes to a couple of hours. The proboscis emerged from the burrow in feeding, as Wilson (1900) had previously noted, with its distal margin facing anteriorly and dorsally towards the substratum to gather food particles and transfer them to the ventral surface where they were glued together with mucus and carried by ciliary currents towards the mouth.

The feeding mechanism of *Ochetostoma* is unknown, except for the observation of Sluiter (1884) that in the shallow water-covered part of the beach at Billiton the proboscides of *O. erythrogrammon* moved slowly on the sand to shovel up sand and organic matter on to the proboscis groove and convey them to the mouth.

In the present study observations were made on *Ochetostoma* specimens both in their natural habitat on the beach and also in the laboratory.

Materials and Methods

Ochetostoma erythrogrammon occurs in large colonies between mean low water neaps and mean low water springs in the intertidal sandy mud of Singapore and neighboring islands. An opening, 3 mm. in diameter, through which the proboscis may emerge in feeding, leads into a U-shaped burrow of 1 cm. diameter. The burrow consists of two vertical or oblique tunnels each 20 cm. long connected by a horizontal tunnel 25–45 cm. long. Although the burrows can be located by the proboscides above the surface at ebb tide, digging up the animals without damage is not easy because of the difficulty of locating the direction of the horizontal tunnel due to blocking up of the other opening of the burrow by a plug of sand or mud.

Sluiter (1884) reported the ease with which the intact animal was obtained by pressing the foraging proboscis on the sand with the fingers and digging up the trunk with the other hand at Billiton. Due presumably to the less muddy substratum around Singapore, this method always resulted in autotomy.

Aquaria were set up by using sandy mud from the same bed from where the specimens were collected. Fine carborundum powder was used to trace the ciliary currents on the proboscis under the binocular microscope.

Results and Discussion

At Pulau Hantu, a sandy island south of Singapore, which rises one meter above the highest spring tide, the substratum from mid-tide level downwards consists of greyish to purplish impervious clay with an overlying layer of coarse muddy sand 2–5 cm. thick. At ebb tide the water retained in the interstices of the sand at the higher shore levels slowly drains along and thus wets the sandy crust above the clay subsoil of the lower shore. At every ebb tide, irrespective of the time of day or night, *Ochetostoma erythrogrammon* feeds by extending its proboscis out of the burrow with the dorsal surface touching the substratum to collect sand and detritus with the dorsally turned distal region of its proboscis. When the surface sand and detritus near the mouth of the burrow are taken up, the proboscis extends further in approximately the same direction. The fully extended proboscis, exceeding 25 cm. in length in some specimens, becomes thin and narrow. Its entire ventral surface is covered with sand grains, detritus and extruded mucus (Fig. 1).

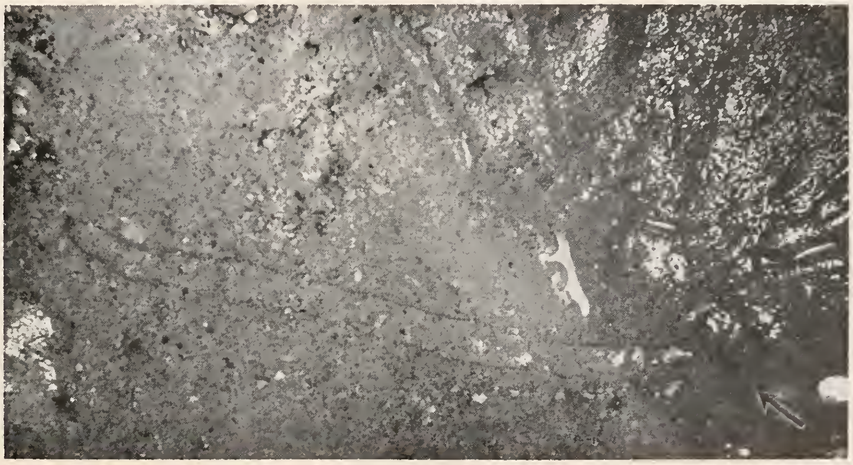

Figure 1. *Ochetostoma erythrogrammon.* The proboscis, stretched horizontally across figure and loaded with sand and detritus, forages on the wet sand outside the burrow (arrow) at ebb tide. Tracks on sand above burrow indicate previous excursions of proboscis.

The smaller particles move towards the mouth along the length of the proboscis but the larger sand grains seem to remain stationary until muscular contractions of adjacent parts of the proboscis move them on. When poked with a stick the fully extended proboscis withdraws, discarding the collected sand grains and detritus at the mouth of the burrow. After an interval of time the proboscis re-emerges to extend in a different direction from the one previously taken. At the end of the low tide several tracks indicating foraging excursions of the proboscis may be seen radiating from the opening of the burrow (Fig. 1).

On more than ten visits to Pulau Hantu during ebb tide proboscides of *Ochetostoma* foraging on the wet sand were observed. That the same specimens protruded their proboscides at every ebb tide is demonstrated by the following observations. During ebb tide at 3:30 A.M. on 27th August 1961 the burrows through which

19 proboscides protruded were marked by iron rods driven into the clay near the opening of the burrow. During the next ebb tide at 5:45 P.M. on the same day 8 proboscides emerged from the marked burrows to forage in brilliant sunshine. They returned into the burrows when rising tide flooded the openings. This method of feeding occurs only when the surface sand is wet enough, since it also occurs on the wet muddy shore of the west coast of Singapore Island, but not on the crumbly, porous, well-drained muddy sand of an adjacent island where a bed of *Ochetostoma* is also found. Stephen and Robertson (1952) also reported the presence of tracks radiating from one side of the opening of *Ochetostoma* burrow on the sandy shore at Mbweni, Zanzibar.

The proboscis underwent frequent changes of shape. It rolled itself up into an almost closed cylinder by apposition of the lateral margins. It flattened out into a long thin ribbon during feeding. It shortened to less than a fifth of the length of the fully contracted trunk or extended to more than four times the length of the relaxed trunk. The width varied between 3 and 11 mm. Observations under the binocular microscope showed that the ventral surface changed frequently from a plane to a concave shape. Moreover, longitudinal troughs and transverse grooves on the ventral surface and puckers along the lateral margins appeared and disappeared in various regions according to the degree of contraction of the underlying muscles.

The cilia lined only the ventral surface of the proboscis and were of uniform length of 11–13 μ in the living state, unlike those of *Echiurus echiurus*, in the proboscis of which Gislén (1940) reported larger cilia on the tip, lateral margins and the "eminence."

As regards the ciliary currents the ventral surface of the proboscis can be subdivided into three regions, namely the distal, middle and proximal regions. The distal region had in its distal 4 mm. or so only posteriorly directed ciliary currents. These also occurred in the middle of its proximal 4 mm., where they were flanked by postero-medially directed ciliary currents (Fig. 2a). During feeding the extremely mobile distal region turned dorsally to explore the substratum and pick up particulate matter. It was also used for digging a burrow.

The middle region, which formed the greater part of the proboscis and varied in length with the degree of extension, had medially directed ciliary currents along the lateral fields. There were posteriorly directed ciliary currents in the middle flanked by postero-medially directed ciliary currents (Fig. 2b).

The proximal region, almost as mobile as the distal region, had a tract of posteriorly directed ciliary currents along the middle. This tract was flanked by a narrow tract of postero-medially directed ciliary currents. Lateral to this was a tract of medially directed ciliary currents. In addition to these there were several narrow tracts of cilia beating laterally outwards along the thickened rim. The rim on each side puckered up into folds and valleys. By referring to the pigments on the rim it was possible to observe that the folds and valleys were not fixed in position but could vary. The valleys at any particular moment could become folds at the next. As a result of this arrangement the movement of particulate matter along the rim could be outward or inward according to whether the outwardly beating or the inwardly beating tracts of cilia were oriented at the top of the fold.

In the laboratory an intact specimen extended its proboscis 4–7 cm. with the

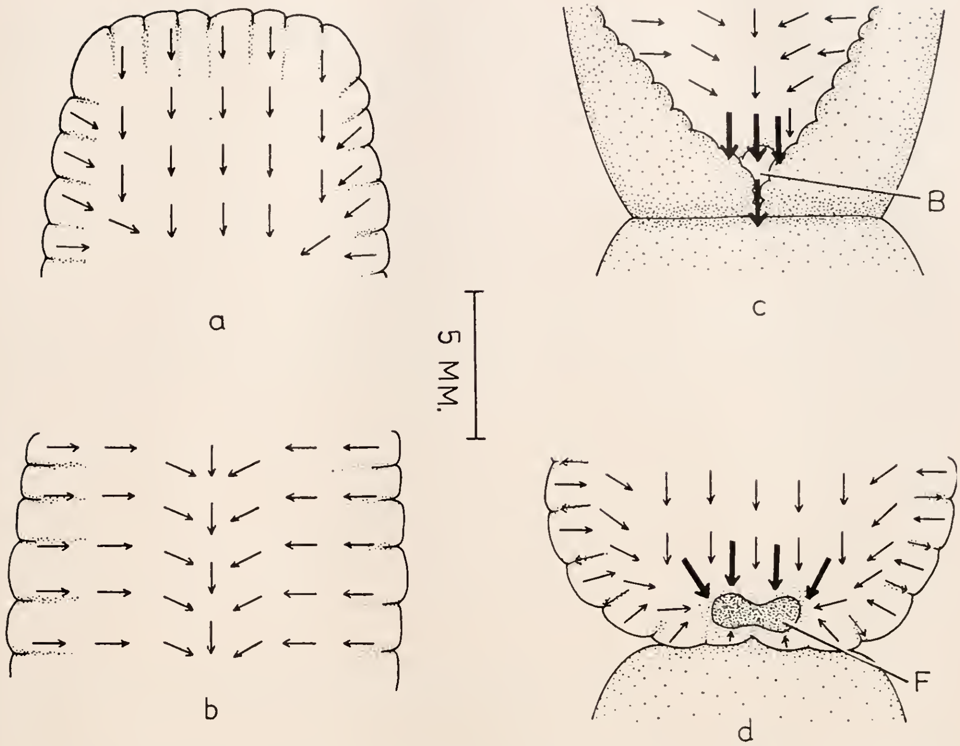

Figure 2. Ciliary currents (small arrows) and paths (large arrows) of accepted and rejected particles on the proboscis of *Ochetostoma erythrogrammon.* a and b, ventral view of distal and middle regions, respectively. c, ventral view of proximal region rejecting particles; B = bulge. d, antero-ventral view of proximal region accepting particles into the mouth funnel (F).

dorsal surface touching the bottom of the waxed tray. The distal part of the proboscis swung from side to side and its ventral surface faced dorsally to scour the substratum and pick up particles of carborundum added. These were coated with mucus and carried in the median tract of posteriorly directed ciliary currents through the expanded proximal region into the mouth (Fig. 2d). When particles of clean sand 1 mm. or more in diameter approached the proximal region, this soon partially rolled up into a cylinder and became dorso-ventrally depressed. A bulge also formed 2–3 mm. anterior to the mouth. The passage into the funnel-shaped proximal region was thus blocked. These large particles therefore moved posteriorly across or alongside the anterior part of the bulge. over the rim of the proboscis ventrally (Fig. 2c) and were rejected.

The equivalent of the bulge of *Ochetostoma* in *Echiurus echiurus* is presumably the ridge or "eminence," since Gislén (1940) found that it could bulge or sink into a furrow. He believed that the peristaltic movements of the eminence helped to move the mucus thread down the mouth-funnel. Two ventral lips or swellings of the proximal region of the proboscis fitted into the depressions on either side of the eminence to prevent larger particles from entering the mouth in *Echiurus echiurus.* In this and in *Ochetostoma erythrogrammon* the rejection mechanism is therefore muscular and differs only in small details. Fisher and MacGinitie (1928) observed that large particles were rejected when the mucus tube was being swallowed by *Urechis caupo* but the details of this rejection mechanism were not described.

Intact specimens placed in aquaria with muddy-sand bottom built U-shaped tunnels by forcing the distal region of the proboscis into the sand and working out a hole with it while the trunk meantime lay prostrate on its side or dorsal surface. The proboscis disappeared into the hole dragging the trunk after it as in *Urechis caupo* (Fisher and MacGinitie, 1928). The ventral setae were not used in digging the hole in *Ochetostoma.* In *Urechis* they were used in enlarging the tunnel by scraping off material from the sides (Fisher and MacGinitie, 1928). *Echiurus echiurus,* however, performed digging movements alternately with the ventral setae at the rate of 6–9 times per minute and the stiffened anterior end of the trunk, while the proboscis remained inactive. Gislén (1940) found that the anterior end of the trunk entered the excavated hole dragging the proboscis along, and illustrated (text-figure 10 at page 15) the posterior end of the trunk and the distal tip of the proboscis remaining outside at one stage of digging a burrow. In spite of the closer systematic relationship between *Ochetostoma* and *Echiurus,* the former resembled more closely the more distant relative *Urechis caupo* in its digging behavior.

When the surface of the aquarium substratum was under 3–4 cm. of water, the proboscis in *Ochetostoma* remained inside the burrow and performed feeding movements by exploring and picking up particles along the wall of the burrow with the distal region of the proboscis. After some time the animal turned around so that the proboscis could explore and collect particles from the other end of the burrow. *Echiurus* also put out part of its proboscis while still submerged under water to collect food particles of the aquarium bottom (Wilson, 1900; Gislén, 1940) and leave distinct tracks (Gislén, 1940). Some time after the water in the aquarium was siphoned out, *Ochetostoma* extended its proboscis out of the burrow to feed in the same manner as observed under natural conditions on the beach. The collection of food particles with the proboscis inside or outside the burrow is presumably the usual method of feeding in all echiuroids, since *Urechis caupo* also gathered sediment with its proboscis while lying outside the burrow in an aquarium (Fisher, 1946). Due to the reduced size of the proboscis in *Urechis caupo,* an alternative method involving filtration of food particles with mucus tube was developed. No mucus tube of the type secreted by *Urechis caupo* was formed in *Ochetostoma.*

SUMMARY

1. *Ochetostoma erythrogrammon* built U-shaped burrows between mean low water neaps and mean low water springs in the intertidal sandy mud of Singapore and neighboring islands.

2. At ebb tide the proboscis emerged from the burrow in wet beaches to collect and swallow sand grains and detritus from the surface of the shore.

3. The ciliary currents on the proboscis and the course of the accepted and rejected particles were described.

4. The feeding mechanism of *Ochetostoma erythrogrammon* was compared with those of *Echiurus echiurus* and *Urechis caupo.*

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SITES OF OXYGEN UPTAKE IN OCHETOSTOMA ERYTHROGRAMMON LEUCKART & RUEPPELL (ECHIUROIDEA)

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In the echiuroid *Urechis caupo* inhalations and exhalations of sea water by the muscular cloacal chamber during respiration occurred through the anus (Fisher and MacGinitie, 1928b). These authors pointed out that the peristaltic movements passing along the trunk of this worm not only renewed the water in its burrow but also moved that in the respiratory chamber of the gut. Redfield and Florkin (1931) observed that in *Urechis* the oxygen in the water enclosed within the burrow and in the blood was insufficient to maintain the normal metabolic rate for the duration of the low tide, during which the hemoglobin of the blood might be expected to transport an adequate supply of oxygen to the organs of the body. Hall (1931) found that the oxygen consumption of *Urechis caupo* was comparable to that of related forms.

Ochetostoma erythrogrammon in many poorly drained beaches in the tropics also feeds during low tide by protruding its proboscis outside the burrow. The reduced availability of oxygen in the burrow at ebb tide, the small diameter of the hindgut, and the irregular, infrequent and small outflows from the anus of specimens in burrows built along the glass wall of the aquarium in the laboratory suggest that the anus may not be the sole organ of respiration.

In the present study the oxygen uptake of entire specimens and of parts of the body of *Ochetostoma erythrogrammon* was determined.

MATERIALS AND METHODS

Specimens of *Ochetostoma erythrogrammon* from the intertidal muddy sand of the west coast of Singapore Island, where feeding also occurs during ebb tide, were starved for 3–5 days to allow the faecal pellets to be completely voided, and their oxygen consumption was determined in a closed bottle. As a precaution against excessive peristaltic movements each specimen was confined in a cylindrical bag 1 cm. in diameter and 6–8 cm. long, to which it was acclimatized for one day. This bag of nylon netting of 81 meshes per sq. cm. was slipped, together with the enclosed specimen, into a bottle of about 175-milliliter capacity, the actual volume of which was previously determined.

The natural sea water used was filtered into a jar with a capacity of 13 liters and thoroughly aerated before it was covered with a thick layer of oil and siphoned into individual bottles containing the experimental animals. To ensure that the water siphoned into the bottles was not in contact with air, the water in the bottle was retained only after an amount of water equivalent to twice the volume of the bottle had passed through. The experiments were carried out at 19.3, 19.5, 19.9

and 20.0° C. ± 0.1° C. Each bottle containing the experimental animal was turned at half-hourly intervals to ensure thorough mixing.

To prevent cloacal respiration a nylon rod of suitable diameter was inserted into the cloaca via the anus and secured by ligating the posterior tip of the trunk around it. This treatment did not seem to adversely affect the specimens even after more than 9 hours of anal blockade, since they survived when the rod was released by cutting away the ligature.

The proboscis was easily detached by gently squeezing with a pair of fine forceps its attenuated junction with the trunk. Autotomy of the proboscis occurs in nature and contraction of the circular muscle of the trunk at this junction prevents bleeding. The detached proboscis continues to move for several days with its cilia still beating. Its oxygen uptake was determined immediately after its separation from the trunk.

The oxygen content was determined by the modification of Fox and Wingfield (1937) of the Winkler method using phosphoric acid. A blank control was run at the same temperature with every batch of water to find out the amount of oxygen consumed by microorganisms present in the sample of filtered water. This amount was very small for the duration of the experiments and was deducted from the amount of oxygen consumed by the specimens. The fixed tissues of *Ochetostoma* comprised the proboscis, body wall with attached nephridia and the gut wall drained of its contents. Wet weight refers to their weight after blotting with filter paper, and dry weight, after drying for 24 hours at 100° C. in an oven. The coelomic corpuscles were not included.

To record the peristaltic movements of the trunk the apparatus used was based on the same principle as the one devised by Wells (1951) but with the following modifications to suit the weak movements of *Ochetostoma,* namely the use of (1) a light rubber bung for float, (2) a weak spring attached to a lever with writing point to counterbalance the float and (3) a plastic U-tube of about 8–10 mm. diameter to exactly fit the trunk diameter of the worm.

To record the quantity of water pumped by the worm the inlet end of the plastic U-tube of an apparatus based on the one used by Hall (1931) for *Urechis* had to be submerged below the surface of the water before any water could be pumped out by the weak peristaltic movements of *Ochetostoma.*

Results and Discussion

The movements of *Ochetostoma* likely to influence the oxygen consumption considerably are peristaltic and antiperistaltic movements of the trunk and the movements of the proboscis. *Ochetostoma erythrogrammon,* the biggest specimen of which barely weighs 10 gm., is a small echiuroid compared with *Urechis.* The peristaltic movements of the trunk, which is less muscular than that of *Urechis,* were weak and did not displace a large enough volume of water. Only 0–25 cc. was pumped irregularly over a period of three hours; this is less than the quantity pumped by *Urechis* in one minute (Hall, 1931). In *Ochetostoma* reared in aquaria in the laboratory peristaltic waves at the rate of four per minute may pass through the trunk. Each peristaltic movement causes a stream of water to issue from the opening of the burrow facing the posterior end of the body. These peristaltic movements serve to renew the water of the burrow for respiratory and feeding purposes.

The tracing in Figure 1A shows a spell of regular peristalsis at the rate of about three per minute, while Figure 1B shows some irregular peristaltic movements occurring in another specimen. A series of peristalses is usually succeeded by a rest period of variable duration.

Hall (1931) showed that the oxygen consumption of *Urechis* in U-tubes bore no consistent relation to oxygen partial pressure at least over the range of 138.2 to 93.3 mm. Hg. The range of oxygen tension encountered by *Ochetostoma* in nature must be considerable from flood tide to ebb tide. Under experimental conditions the range of 5.08 to 3.20 cc. oxygen per liter at the onset of the experiments is within the usual range encountered by the animals and the experiments were continued until the oxygen consumed amounted to about 25% of the original amount except in specimen 12 where the experiment was continued until the oxygen content dropped to 1.1 cc./liter. In this case the consumption was not far below the mean, indicating that a fall in oxygen tension did not materially affect oxygen uptake.

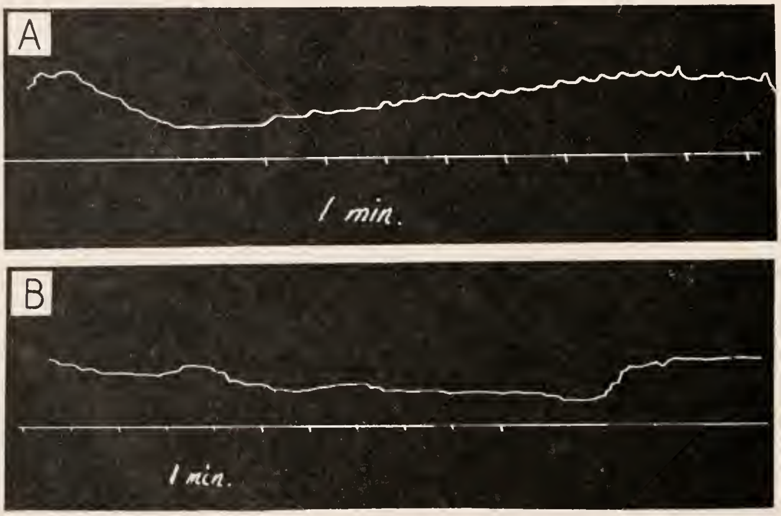

FIGURE 1. Record of peristaltic movements of *Ochetostoma erythrogrammon*. Read from left to right. Time: one division per minute. Each convex (upward) part of the curve represents the passage of a peristaltic wave along the trunk. A. Record of a specimen with regular peristaltic movements. B. Record of another specimen with peristaltic movements occurring at irregular intervals.

In Table I the blocked anus of specimens 1–8 was released at the start of the second period. In specimens 9–12 the anus was blocked at the start of the second period. With the exception of specimens 8 and 9, the rate of oxygen uptake was greater during the second period irrespective of the state of the anus and the oxygen tension. Since Hall (1931) showed that the oxygen consumption in *Urechis caupo* almost doubled with increased activity, presumably there was a tendency towards increased activity during the second period, making it difficult to assess the true effects of blocking the anus. Although the peristaltic movements of *O. erythrogrammon* were subdued by confinement in a nylon bag, it was not possible to

TABLE I

Rate of oxygen consumption of Ochetostoma erythrogrammon

	Specimen			Animal with blocked anus			Normal animal		
	Weight in grams		Duration per period	Initial O_2 content	Oxygen uptake based on		Initial O_2 content	Oxygen uptake based on	
No.	wet	dry			wet weight	dry weight		wet weight	dry weight
			hrs.	*cc./liter*	*cc./gm./hr.*	*cc./gm./hr.*	*cc./liter*	*cc./gm./hr.*	*cc./gm./hr.*
				Period I			Period II		
1	0.5585	0.0845	2	4.32	0.0710	0.4691	4.00	0.0715	0.4725
2	0.9024	0.1440	2	4.32	0.0580	0.3633	4.00	0.0635	0.3802
3	0.6577	0.0947	4	4.84	0.0512	0.3556	4.82	0.0633	0.4395
4	0.5041	0.0939	4	4.84	0.0761	0.4084	4.80	0.0962	0.5163
5	0.8646	0.1437	4	4.84	0.0571	0.3436	4.80	0.0660	0.3969
6	0.6347	0.1158	4	4.84	0.0618	0.3388	4.74	0.0827	0.4531
7	0.5965	0.1025	4	4.84	0.0826	0.4808	4.74	0.1030	0.5993
8	0.5928	0.0772	4	4.84	0.0613	0.4704	4.74	0.0513	0.3943
				Period II			Period I		
9	1.5515	0.2497	4	5.08	0.0358	0.2225	4.49	0.0369	0.2295
10	0.6624	0.1085	4	5.08	0.1330	0.8120	4.49	0.1021	0.6231
11	0.7096	0.1145	4	4.85	0.1134	0.7031	4.49	0.0539	0.3343
12	0.9370	0.1621	9½, 8½	4.64	0.0697	0.4031	4.00	0.0542	0.3135
Mean uptake:					*0.07258*	*0.4476*		*0.07038*	*0.4294*

ensure that the activity was of equal intensity between the various experimental periods.

In cases where the oxygen uptake fell after blocking of the anus, the fall was small, however, indicating that the cloaca and hindgut in *O. erythrogrammon* are of no respiratory significance in contrast to *Urechis caupo* (Fisher and MacGinitie, 1928b; Redfield and Florkin, 1931).

Hall (1931) pointed out the large amount of blood present in *Urechis* and its inclusion in the weight of tissues in the calculation for the rate of oxygen uptake would give a low value. Similarly, in 10 specimens of *O. erythrogrammon* the blood and gut fluid averaged 82% of total weight of the animal against 35% for *Urechis caupo* (Hall, 1931) and were not included in the calculation of oxygen consumption.

Table II shows that both trunk and proboscis, when separated from each other, consumed oxygen. With the exception of specimens 1 and 14, the trunk consumed more oxygen than the entire animal during the first experimental period, thus demonstrating the variability of oxygen uptake and establishing the trunk as the chief respiratory organ in this species. The higher uptake of the proboscis-less trunk was probably due to increased activity of the trunk after the loss of the proboscis. Hence in addition to its role in pumping and renewing the water in the burrow for respiratory and feeding purposes, the trunk of *O. erythrogrammon* also serves as a respiratory surface for oxygen uptake, for which it is well suited be-

TABLE II

Rate of oxygen consumption of the trunk and proboscis of Ochetostoma erythrogrammon in cc./gm./hr.

Specimen No.	Trunk wet weight	Proboscis wet weight	Duration of period	Period I: Normal animal			Period II: Trunk separated from proboscis				
				Initial O_2 content	Oxygen uptake based on		Initial O_2 content	Trunk O_2 uptake based on		Proboscis O_2 uptake based on	
					wet weight	dry weight		wet weight	dry weight	wet weight	dry weight
	gm.	*gm.*	*hrs.*	*cc./liter*	*cc.*	*cc.*	*cc./liter*	*cc.*	*cc.*	*cc.*	*cc.*
1	0.3721	0.1864	2	4.00	0.0715	0.4725	3.40	0.0976	0.5430	0.0458	0.4853
2	0.5678	0.3346	2	4.00	0.0635	0.3802	3.40	0.1212	0.6226	0.0700	0.6996
3	0.3963	0.2614	2	3.32	0.0544	0.3781	3.20	0.1007	0.5443	0.0365	0.4455
4	0.4515	0.0526	2	3.32	0.0786	0.4222	3.20	0.1198	0.6152	0.1131	0.9916
5	0.6651	0.1995	2	3.32	0.0341	0.2054	3.20	0.0576	0.3015	0.0158	0.1910
6	0.5105	0.1242	2	3.32	0.0669	0.3666	3.20	0.0886	0.4416	0.0843	0.7817
13	0.4454	0.2102	2	4.32	0.0719	0.4457	4.00	0.1313	0.6667	0.0545	0.6367
14	0.5174	0.2741	3	3.90	0.1302	0.8280	4.89	0.1513	0.8062	0.0463	0.4627
15	0.8285	0.3020	3	3.49	0.0345	0.2400	5.08	0.0507	0.3073	0.0453	0.5240
Mean uptake:					0.0672	0.4123		0.1021	0.5387	0.0568	0.5798

cause of the following reasons: firstly, the thinness of the body wall, which is a common feature of the genera *Ochetostoma* and *Thalassema*, facilitates diffusion of oxygen. Secondly, the large surface area is further increased by elongation of the trunk usually seen in specimens inside the burrows in laboratory aquaria. Thirdly, the presence of a large quantity of body fluid and haemoglobin-containing coelomic corpuscles continually agitated by peristaltic movements.

The oxygen uptake of the detached proboscis in 9 specimens averaged 17.2% (range: 7.6–25.4%) of the combined uptake of detached trunk and proboscis. The proboscis is therefore an accessory but not indispensable respiratory organ, since proboscis-less trunks survive indefinitely. Because of the extensibility of both trunk and proboscis it is difficult to compare their available respiratory surface. Due to the different degrees of hydration between body wall and proboscis, the average oxygen uptake of the trunk was twice that of the proboscis per gm. wet weight, although on the basis of dry weight the average uptake was approximately equal (Table II).

It is obviously an advantage to the proboscis in being able to respire independently of the trunk, since at ebb tide when the proboscis is fully extended foraging on the surface of the wet sand its actively moving distal tip is some 25 cm. away from the trunk that lies in the oxygen-depleted water inside the burrow. The presence of a certain amount of coelomic fluid and coelomic corpuscles inside the proboscis during full extension presumably increases its efficiency as a respiratory organ. This respiratory function explains the survival of the proboscis several days after its severance from the trunk.

An indirect evidence in support of the respiratory function for the proboscis is the length and extensibility of the proboscis in the genus *Ochetostoma* and the presence of gill-like processes along the ventral margins in the proximal part of the proboscis in *O. arkati* (Prashad, 1935; Wesenberg-Lund, 1959) and *O. atlantidei* (Wesenberg-Lund, 1959). These processes, Wesenberg-Lund (1959) suggested, may function as a respiratory organ. The large oxygen uptake by the proboscis in *O. erythrogrammon* suggests respiratory function for the entire available external surface of the proboscis. It would be interesting to know whether the outer row of processes in *O. atlantidei* would disappear with full extension of the proboscis; the inner row in *O. atlantidei* and the processes in *O. arkati* may well be mere folds and presumably disappear with full extension, since in *O. erythrogrammon* similar folds or processes occur transiently along the ventral margin when the proboscis contracts but disappear with full extension.

Although a study of the relative importance of the different sites of oxygen uptake in *Urechis caupo* is lacking, available evidence, such as the presence of a long, large, inflatable hindgut and cloaca (Fisher and MacGinitie, 1928a; Fisher, 1946) and the occurrence of inhalations and exhalations through the anus (Fisher and MacGinitie, 1928b; Redfield and Florkin, 1931; Hall, 1931; Fisher, 1946), points to the importance of the hindgut as a respiratory organ. Redfield and Florkin (1931) observed antiperistalsis of *Urechis* hindgut, obtained 25–35 cc. of water at a single discharge during anal exhalation and found that this water contained less oxygen but more carbon dioxide than aquarium water outside the body. They believed that the thick body wall of *Urechis* must absorb a relatively small amount of oxygen in comparison with the hindgut. The relative importance of the sites of respiratory exchange thus differs between the echiuroids,

TABLE III

Rates of oxygen consumption of some annelids, echiuroids and a sipunculoid

Animal	Author	Oxygen consumption *cc./gm./hr.*
Tubifex	Brazda (1939)	0.2
Schizobranchia insignis	Dales (1961)	0.1920
Ochetostoma erythrogrammon	Present author	0.0692
Bispira voluticornis	Zoond (1931)	0.0573
Sabella	Wells (1952)	0.0488
Lumbricus terrestris	Johnson (1942)	0.045
Myxicola	Wells (1952)	0.0398
Sipunculus nudus	Cohnheim (from Krogh, 1916)	0.0313–0.0688
Arenicola marina	Borden (1931)	0.031
Nereis virens	Bosworth, O'Brien and Amberson (1936)	0.026
Hirudo	Heilbrunn (1952)	0.023
Urechis caupo	Hall (1931)	0.0198
Glycera siphonostoma	Montuori (from Krogh, 1916)	0.0146
Chaetopterus pergamentaceus	Bosworth, O'Brien and Amberson (1936)	0.0078

Urechis and *Ochetostoma.* Presumably, in *Urechis* the thick body wall prevents rapid diffusion of oxygen and the small size of the proboscis offers only a small respiratory surface. Apart from members of the genus *Urechis,* only *Nellobia eusoma* has a large hindgut and cloaca (Fisher, 1946), which may have a respiratory function. All other known echiuroids have a slender coiled hindgut and small cloaca, which are obviously not adapted for efficient respiratory function, but they have a thin and presumably respiratory body wall.

Krogh (1916) pointed out the difficulty of comparing the metabolism of different invertebrate animals and that a fair comparison could not be made on the basis of fresh weight because of the enormous differences in the composition of the various animals. Although dry weight offers a better basis for comparisons, the presence of varying amounts of reserve material, skeletal and other inactive tissues also renders this far from ideal (Krogh, 1916). When the oxygen consumption of *O. erythrogrammon* based on wet weight of fixed tissues (*i.e.* minus coelomic fluid and gut fluid) is compared with annelids, sipunculoids and *Urechis,* it occupies a position near the top in the descending series of rates shown in Table III.

SUMMARY

1. The oxygen consumption of *Ochetostoma erythrogrammon* averaged 0.0692 cc. per hour per gram of wet weight of fixed tissue.

2. After blockade of the anus the oxygen uptake did not diminish, indicating that the cloaca and hindgut have no significant respiratory function.

3. Both trunk and proboscis took up oxygen after separation, the latter consuming oxygen averaging 17% of the combined uptake of trunk and proboscis.

4. The relative importance of cloaca and hindgut, trunk, and proboscis as respiratory organs was discussed.

5. The oxygen consumption of *O. erythrogrammon* was compared with related animals.

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DAY-LENGTH AND TERMINATION OF PHOTOREFRACTORINESS IN THE ANNUAL TESTICULAR CYCLE OF THE TRANSEQUATORIAL MIGRANT DOLICHONYX (THE BOBOLINK)[1]

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At the end of a reproductive season the testes of birds undergo a regression, which results in minute, inactive gonads composed of small tubules formed almost entirely of spermatogonia, the tubules separated by masses of undifferentiated interstitial tissue. In those passeriform birds in which testicular recrudescence is under photoperiodic control, periods of long day-length fail not only to prevent the occurrence of this regression but fail to stimulate recrudescence after the regression is completed. Hence, this period of the annual cycle is known as the *photorefractory phase* (the "preparatory phase" of Wolfson, 1958, p. 372). After some weeks of exposure to short photoperiods this refractoriness disappears; thereafter, long photoperiods again stimulate the mechanism which produces testicular recrudescence.

Among transequatorial migrants the existence of a photoperiodic mechanism, including a refractory period, has so far been demonstrated only in the bobolink, *Dolichonyx oryzivorus* (Engels, 1959, 1961; Wolfson and Westerhoff, 1960). It has been shown (Engels, 1961) that (1) exposed to the natural day-lengths of the northern hemisphere, as experienced by such temperate zone migrants as *Junco hyemalis* and *Zonotrichia albicollis,* the testicular cycle of *Dolichonyx* develops ultimately (April) approximately in normal phase; (2) *Dolichonyx* is able to overcome naturally induced, autumnal refractoriness on longer photoperiods (12 hours) than can at least some populations of *Junco* and *Zonotrichia;* but (3) the rate of response to long photoperiods (14 hours) following termination of refractoriness is slower in *Dolichonyx* than in the other two forms. However, as was pointed out (Engels, 1961, p. 146), the photoperiods used in these earlier experiments to release refractoriness were considerably shorter, and the duration of treatment considerably longer, than birds could be expected to experience in nature in a migration from the northern to the southern hemisphere soon after the September equinox.

The experiments now to be reported upon were designed (1) to compare *Dolichonyx* to north temperate zone migrants with respect to the timing of termination of refractoriness when exposed to the natural day-lengths of the north temperate zone, and (2) to determine the capacity of *Dolichonyx* to overcome refractoriness when exposed to photoperiods more nearly comparable to those normally experienced in post-nuptial transequatorial migration. Since only meager and scattered information on the timing of the southward transequatorial passage of *Dolichonyx* can be found in the literature, special effort was made to establish the

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[1] Research supported in part by a grant from the National Science Foundation (G-6163).

pertinent facts. The details which are presented below on autumnal migration in South America were obtained mostly from specimens in the collections of major museums in the United States.

MATERIALS AND METHODS

Twenty-one adult male bobolinks (*Dolichonyx oryzivorus*), all of which had experienced the natural day-lengths of the northern hemisphere during the preceding summer, were used in the experiments. Two were captured near Wilmington, North Carolina, in September, a few weeks before experimental treatment was begun ("autumn captures"); fifteen were captured near Gainesville, Florida,[2] in early May of the year of experimental treatment ("spring captures"); four had been in captivity one to two years ("second-year experimentals").

The birds were kept in an outdoor aviary, exposed to the natural day-lengths of Chapel Hill (Lat. 36° N.), until experimental illumination was begun at various times from October 2 to November 28. At the beginning of artificial lighting they were confined individually in small cages (each about 22 cm. × 25 cm. × 40 cm.) Each cage was furnished with a food hopper and two 100-cc. water-tubes. Food consisted of a mash formulated as a complete diet for egg-laying "game" birds; a small amount of soluble terramycin was added to the water. The lights used to provide the experimental photoperiods were automatically switched on and off by electrically operated time-switches. Eight different lighting schedules were used; details of the schedules, including light intensity, are given below. Light intensity was measured at perch-level.

The birds were examined weekly. Testicular recrudescence was determined by the development of black pigment in the beak, especially evident in the "mandible"; this pigmentation is caused directly by the male sex hormone (Engels, 1959). In seven bobolinks which were killed, during the winter of 1961–62, within a few days to a maximum of two weeks following the first appearance of this pigmentation, the testes averaged 179 mm.^3 per bird in volume (range, single testis, 32.5 mm.^3 to 131.5 mm.^3) (previously unpublished data). The volume of an inactive testis, in males with light-colored beaks, is less than 2 mm.^3

RESULTS

1. *Termination of refractoriness under natural day-lengths of Lat. 36° N. (Figure 1)*

Five groups of birds, two to four in each group, were used in this series of experiments. *Group A* (Group E of Engels, 1961, p. 143) consisted of two "autumn captures" removed from the aviary to an indoor, light-tight compartment on October 2 and exposed thereafter to constant daily 14-hour photoperiods (white fluorescent lights, intensity about 90 foot-candles.) Neither of these birds had developed beak pigmentation by late May, when observations were terminated.

Group B consisted of four birds, all "2nd year experimentals." They were exposed to natural day-lengths from late May until November 28, after which white

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[2] This study could not have been made at this time except for the kindness of Cameron E. Gifford, University of Georgia (presently at Earlham College, Richmond, Indiana), who generously made these birds available to me after I had failed in attempts to capture some in North Carolina during the spring migration of 1961.

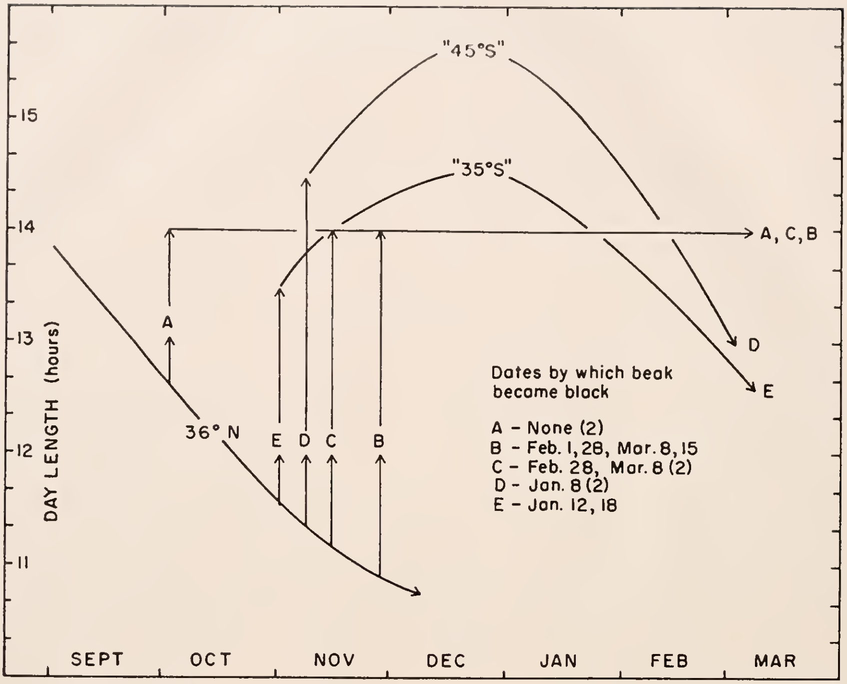

FIGURE 1. Experiments to determine the time of termination of photorefractoriness in bobolinks exposed to the natural day-lengths of Lat. 36° N. At various times between October 2 and November 28, day-length was abruptly increased either to 14 hours daily or to a simulation of the daily change in day-length (sunrise-sunset) occurring at that time in the southern hemisphere. For this treatment groups A and E were moved, at the times indicated, to light-tight compartments indoors; the other groups remained outdoors, where the artificial lighting was superimposed on the natural day-length. The development of black pigment in the beak is evidence of testicular recrudescence.

fluorescent lights (which insured a minimum intensity of 30 to 35 foot-candles) provided 14-hour daily photoperiods (5:15 AM–7:15 PM); these birds remained in the outdoor aviary, hence during the dark period were exposed to approximately normal light of the night sky. Beak pigmentation indicative of testicular recrudescence developed first in one bird during the week ending February 1, in the other three before March 15.

Group C consisted of three "spring captures"; they were subjected to exactly the same light schedules and other conditions as Group B except that the 14-hour photoperiods were begun almost two weeks earlier, on November 15. Beak pigmentation developed during the last week of February and first week of March.

Group D consisted of two "spring captures"; they were caged in an outdoor aviary where, beginning November 8, incandescent lamps (intensity about 45 foot-candles) provided photoperiods which approximated the changing sunrise-sunset day-lengths normally occurring during the months of November to March at Lat. 45° S. The abrupt change in photoperiod on the first day was from $11\frac{1}{3}$ hours to $14\frac{1}{2}$ hours; the photoperiod then increased gradually to more than $15\frac{1}{2}$ hours in

December. Both birds developed the nuptial pigmentation of the beak during the first week of January. (These lights were automatically switched on and off by an "astronomical-dial" time switch, geared to the daily changes in time of sunrise and of sunset at Lat. 45°, manufactured by the Sangamo Electric Co., Springfield, Illinois.)

Group E also consisted of two "spring captures." They were removed from an outdoor aviary on November 1 and thereafter subjected indoors to photoperiods corresponding to the changing sunrise-sunset day-lengths of Lat. 35° S. Beak pigmentation developed during the second and third weeks of January. (White fluorescent lights, intensity about 90 foot-candles, switched on and off by an "astronomical-dial" clock were used; between photoperiods the birds were in absolute darkness.)

General conclusion

The mechanism which stimulates testicular recrudescence in bobolinks is refractory to long photoperiods in early autumn, at least until October 1; when birds are held captive in the northern hemisphere, exposed to the natural day-lengths of Lat. 36° N., photorefractoriness is terminated sometime during October, definitely by November 1.

2. *The southward migration of bobolinks and the day-lengths experienced by them during migration (Figures 2, 3)*

Oberholser (1920) brought together data from a number of localities in the United States, gathered over various periods of years, which give for each locality an average "first date seen" and an average "last date seen," that is, average dates of arrival and departure. These data for the eastern United States in autumn are incorporated in the accompanying chart of latitudinal distribution (Fig. 2). Since 1947 "Audubon Field Notes," in an annual review of autumnal migration of North American birds, provides some additional data for points within the United States. Unfortunately, records chiefly only of an unusual nature (exceptionally early or exceptionally late observations) are published here, but there have been a few notices on peaks of abundance. All the "Audubon Field Notes" records, through 1960, are also represented in Figure 2. No such data are available for the migration south of the United States. Through the kind cooperation of a number of individuals and institutions I have been able to locate, in museums of this country, 89 specimens taken south of the United States and to compile the data on locality and date of collection.[3] Thirty-seven of these specimens were taken in September, October, and November. Together with the Oberholser and the "Audubon Field

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[3] I am grateful to the following individuals and institutions for lending specimens for my personal examination and/or for supplying the "label data" on specimens not seen by me: Dean Amadon, American Museum of Natural History (New York); Kenneth C. Parkes, Carnegie Museum (Pittsburgh); Emmet R. Blake, Chicago Natural History Museum; R. A. Paynter, Jr., Museum of Comparative Zoology (Harvard); Harrison B. Tordoff, Museum of Zoology, University of Michigan; James Bond, Philadelphia Academy of Sciences; and P. S. Humphrey and Mary A. Heimerdinger, Peabody Museum of Natural History (Yale).

The following reported that their collections lacked specimens of *Dolichonyx* taken south of the United States: Alden H. Miller, Museum of Vertebrate Zoology, University of California; Donald Hoffmeister, Natural History Museum, University of Illinois; E. Raymond Hall, Natural History Museum, University of Kansas; George H. Lowery, Museum of Natural Science, Louisiana State University; and H. G. Deignan, U. S. National Museum (Washington).

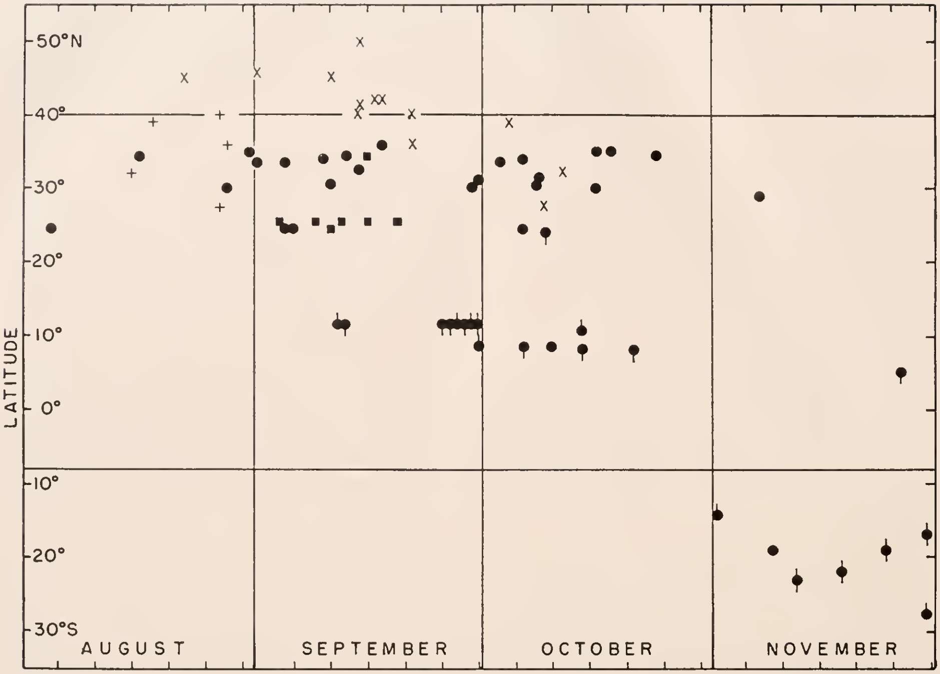

FIGURE 2. Latitudinal distribution of bobolinks from August through November. The horizontal lines approximate the southern border of the breeding grounds (Lat. 40° N.) and the northern border of the "wintering" grounds (Lat. 8° S.). Plain circles are from published sight records, mostly from *Audubon Field Notes* (vols. 1–14, 1947–1960); squares indicate "peaks of abundance" reported in the same journal; + and × indicate annual average dates of arrival and departure, respectively (average dates of "first seen" and "last seen"; data from Oberholser, 1920); circles with vertical lines indicate museum specimens (line above circle = male, below = female). The major part of the "wintering" grounds lie below about Lat. 14° S. (in Peru, Bolivia, Brazil [Matto Grosso], Paraguay and northern Argentina), but the species occurs regularly between November and March in the Trujillo and Lima districts of Peru, on the western slope of the Andes, at Lat. 8°–12° S. (Koepcke, 1961).

Notes" data, these records give a reasonably clear picture of the southward migration of bobolinks (Fig. 2). The breeding grounds, north of Lat. 40° N., are usually emptied by the end of the second or third week of September. Meanwhile, some individuals (which must have started their journey in August) have at that time already made the trans-Gulf or trans-Caribbean passage and are in Central or South America at Lat. 8°–11° N. Although some individuals may still be in the United States in late October, or exceptionally even in early November, some have reached the "wintering" area below Lat. 8° S. at least by November 1. In Figure 3 the same latitudinal distribution data are plotted against day-length (sunrise-sunset plus morning and evening civil twilight). The arrow drawn through this figure gives a rough approximation of the day-lengths experienced by an "average" bobolink during the southward transequatorial migration. Whatever the degree of validity of this approximation, there are certain limits on the day-length cycle which migrating bobolinks may experience, limits which are imposed

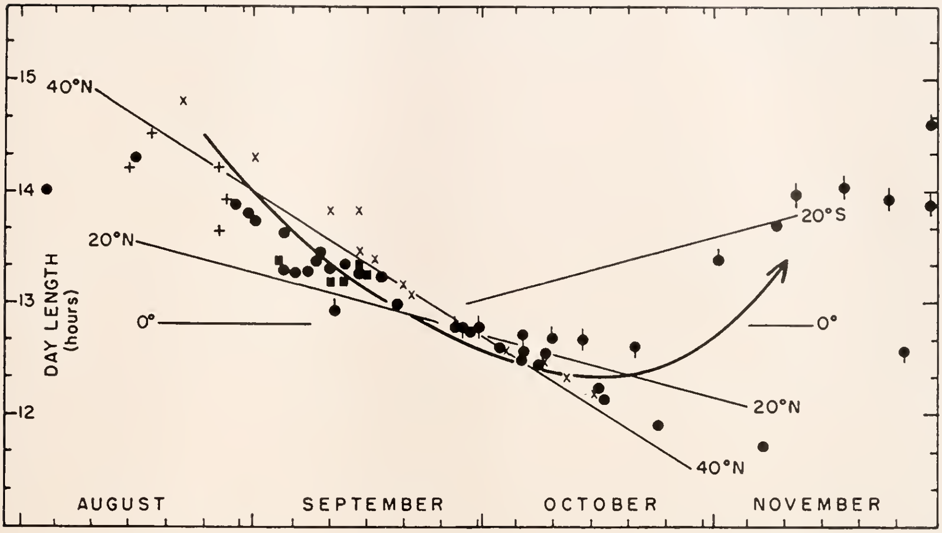

Figure 3. Day-lengths (including civil twilight) experienced by bobolinks from August through November. Same data and symbols as in Figure 2. The arrow roughly approximates the migration of an "average" bobolink. See text for discussion of limiting factors. (Day-length data from: Tables of Sunrise, Sunset and Twilight: Supplement to the American Ephemeris, 1946; U. S. Naval Observatory. Government Printing Office, Washington, D. C.)

by the changes in day-length at different latitudes, some of which also are shown in this figure. Any birds which might reach the equator during the third week of September would experience at about that time their shortest day-length, about 12 hours 45 ± 4 minutes (sunrise-sunset plus civil twilight); thereafter they would be exposed to gradually increasing day-lengths (until the December solstice). Any birds still north of the equator on October 1, at whatever latitude, would at that time also experience day-lengths of about 12 hours 45 ± 4 minutes. Day-lengths continue to decline in the northern hemisphere until the December solstice but at a progressively lower rate the lower the latitude. As a consequence of this phenomenon, and as is evident in Figure 3, a southwardly migrating bird begins to experience a progressive *increase* in day-length while still north of the equator in October, an even greater increase in November; it is again exposed to a little more than 12¾ hours of day-light when it reaches the equator. South of the equator, of course, day-lengths then everywhere are increasing above that level.

3. *Termination of refractoriness under day-lengths comparable to those experienced in transequatorial migration* (*Figure 4*)

In addition to the previously described Group A, three groups of bobolinks, three birds in each group, were used in this series of experiments. All of these nine birds were "spring captures" which had spent the summer in an outdoor aviary, exposed to the natural day-lengths of Lat. 36° N. As with Group A, they were brought indoors at the beginning of October into light-tight, ventilated compartments and exposed there to artificial photoperiods under white fluorescent lights. Between photoperiods they were in absolute darkness.

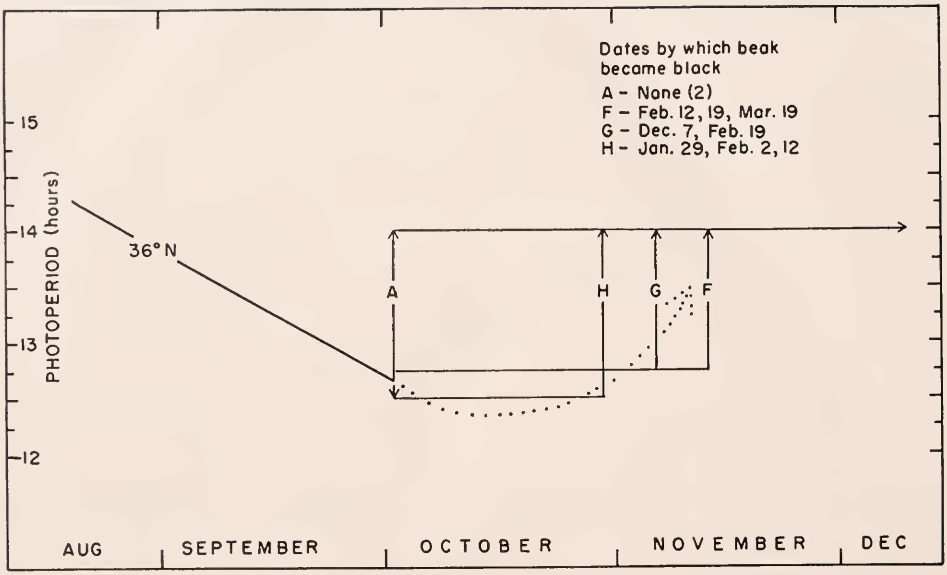

FIGURE 4. Experiments to determine length of photoperiod, and duration of treatment, which may release bobolinks from photorefractoriness during October and November. On October 2 ten birds were removed, from the natural day-lengths (including civil twilight) of Lat. 36° N., to constant daily photoperiods of different lengths, in four groups. Development of black pigment in the beak indicates testicular recrudescence. The dotted line is a rough approximation of day-length experienced by an "average" bobolink during southward, post-nuptial migration (*cf.* Figure 3).

Group F

These three birds were exposed to $12\frac{3}{4}$-hour photoperiods (light intensity about 90 foot-candles) for six weeks; on November 13 the photoperiod was increased to 14 hours. Two birds developed beak pigmentation in early and mid-February, the last in the third week of March.

Group G

As in the previous group the initial photoperiods were $12\frac{3}{4}$ hours (light intensity 90 foot-candles), but these were continued for only five weeks; the 14-hour photoperiods were begun on November 6. One bird developed beak pigmentation surprisingly early, during the first week of December, another not until the third week of February. The third bird accidentally hung itself in the cage during the period December 22–26. At the time the accident was discovered the lower beak was discolored, but the testes were minute.

Group H

The initial photoperiods were $12\frac{1}{2}$ hours (light intensity about 45 foot-candles) and they were continued for only four weeks. On October 30 the photoperiods were increased to 14 hours. One bird developed beak pigmentation during the last week in January, the other two in early February.

General conclusion

The post-nuptial photorefractoriness exhibited by bobolinks at the beginning of October can be terminated by only five weeks of relatively long, $12\frac{3}{4}$-hour, photoperiods or by only four weeks of 12½-hour photoperiods. These lighting schedules approximate the day-lengths experienced by bobolinks in post-breeding migration.

Discussion

Bartholomew (1949) pointed out that photorefractoriness might play an important role in regulating the timing of the annual recrudescence of the testes in *Passer domesticus.* It may be a significant factor in many passeriform birds in which the annual testicular cycle is controlled by photoperiodism (Wolfson, 1952; Wolfson, 1958). It assures that the gonad-stimulating mechanism does not again become activated, following regression and reconstitution of the inactive testes, until middle or late autumn when days are short, and continue to shorten, and the photoperiodic stimulus is therefore weak at best. Recrudescence of the testes, and the appearance of male sexual behavior, with ultimate development and release of motile spermatozoa, is thus suitably delayed.

In the absence of experimental evidence it was not easy to fit transequatorial migrants into this picture, because in post-nuptial migration they pass directly from the shortening days of the northern hemisphere autumn into the lengthening days of the southern hemisphere spring, never experiencing the retarding effect of the short days of winter. This consideration leads logically to the question of the photorefractory phase in such migrants. Bissonnette (1937) had suggested that "prolonged refractory periods . . . would supply the necessary delay to prevent even transequatorial migrants from breeding in their southern range" (p. 263). Farner (1954) postulated for transequatorial migrants "a characteristically longer refractory period" (p. 29). Wolfson (who since 1958 has preferred the term "preparatory phase") has spoken of the relation between day-length and the photorefractory phase as the "main problem" in equatorial and transequatorial migration and of the regulation of this phase as the "critical problem" (Wolfson, 1959, pp. 706–7; Wolfson, 1960, p. 785). Wolfson and Westerhoff (1960), in a report on some preliminary experiments with bobolinks, suggested that in this species, as compared with temperate zone species, a *longer period* of short days may be required in the regulation of the preparatory phase.

In all temperate zone species so far investigated, the refractory period is terminated in nature in middle to late autumn, that is, variously between mid-October and mid-November or even early December (published data summarized by Farner, 1954; Farner, 1959; Wolfson, 1958). It seems evident from our first series of experiments that, under comparable conditions (*i.e.,* the natural day-lengths of middle latitudes in the northern hemisphere), photorefractoriness in *Dolichonyx* may be terminated as early as November 1. Since normal reproductive activity in the preceding season had been suppressed in these captive birds, testicular regression may have been accelerated, leading possibly to an earlier termination of refractoriness. However, in some temperate zone species refractoriness persists until mid-November and in at least one species until early December (*Zonotrichia albicollis,* Lat. 42° N., Shank, 1959). Thus, even if we allow two to five weeks for a possible effect of the celibacy imposed on our captive birds, it would seem that *Dolichonyx* does not differ markedly from temperate zone migrants with respect to the timing of release from refractoriness in the northern hemisphere autumn.

The present experiments also permit comparison of *Dolichonyx* and temperate zone migrants in two other respects, namely, the length of the short days effective in terminating refractoriness, and also the number of such short days required. In previously reported experimental studies on the regulation of photorefractoriness by short days, photoperiods longer than 12 hours have not been employed (except by Wolfson and Westerhoff, 1960, for *Dolichonyx*). However, in at least some populations of *Junco hyemalis* and *Zonotrichia albicollis,* even eight weeks of exposure to 12-hour photoperiods, beginning October 1, does not release refractoriness (Engels, 1961). In another population of *Z. albicollis,* studied by Shank (1959), five weeks of 12-hour photoperiods, beginning October 1, failed to terminate refractoriness in any of nine males tested; thirty-one days of 9-hour photoperiods failed for five of eight males tested. In the present experiments, refractoriness in *Dolichonyx* was terminated by only four weeks of 12½-hour photoperiods, and also by five weeks of 12¾-hour photoperiods, both beginning October 1. Therefore, the suggestion of Wolfson and Westerhoff, mentioned above, that *Dolichonyx* may require a longer period of short days to complete the "preparatory" phase, seems to be inapplicable. (Termination of refractoriness by November 1 under the influence of natural day-lengths at Lat. 36° N. also argues against this idea.) It may be suggested rather that in *Dolichonyx* one of the adjustments of the photoperiodic mechanism to transequatorial migration lies in the capacity to overcome refractoriness on relatively longer days, up to at least 12¾ hours.

Another adjustment, to the long days experienced between breeding seasons during the southern hemisphere summer, was indicated by earlier studies (Engels, 1961) which showed that, when bobolinks, juncos and white-throated sparrows were subjected to identical effective treatment for termination of refractoriness and stimulation of the gonad, testicular recrudescence in the bobolinks lagged several weeks behind recrudescence in the other two forms. It was suggested, at the time (p. 145), that this retardation of the bobolink cycle might be explained simply as evidence of a very slow rate of response (to 14-hour photoperiods) following the termination of refractoriness. An interesting alternative explanation might be that, during the period of exposure to shorter days, the photorefractory phase of *Dolichonyx* is not actually terminated (Engels, 1959, p. 764) but reaches a point where "longer days [no longer] prevent, but perhaps delay [its] completion" (Wolfson, 1960, p. 785). It was hoped initially that the present experiments would throw light on this question but, among other deficiencies, the number of birds used was too small to give the required information. It will be interesting to test the idea with experiments of a different design.

Examination of museum specimens indicates that the black pigmentation of the beak, which we used as a criterion of testicular recrudescence in bobolinks, in nature does not develop until April, when northward migration already is underway. Thus, in all of our experiments the development of this pigmentation was greatly accelerated, occurring in December, January, February or early March, even when we attempted to approximate, after October 1, the day-lengths expected to be encountered during the autumnal migration, followed by an approximation of the average day-length of the southern hemisphere summer. Obviously, much remains to be learned about the regulation of the natural timing of the testicular cycle in this transequatorial migrant.

SUMMARY

1. The testicular cycle of *Dolichonyx oryzivorus,* a bird which breeds above Lat. 40° N. and winters below Lat. 8° S., exhibits a photorefractoriness in early autumn, which is maintained by constant daily 14-hour photoperiods (experiment begun October 2).

2. Some individuals which had been held captive outdoors, exposed to the natural day-length of Lat. 36° N., were shifted to experimental, long photoperiods at various times between November 1 and November 28. Within a few months all of them developed the characteristic black beak pigmentation indicative of testicular recrudescence. From these results it is concluded that, under the influence of autumnal day-lengths of middle latitudes of the northern hemisphere, refractoriness is terminated by November 1 and that *Dolichonyx* thus does not differ appreciably from temperate zone species in the timing of this event under these conditions.

3. From published data on the time of autumnal migration within the United States and from data furnished by museum specimens collected south of the United States, an approximation of the cycle of day-lengths experienced by migrating bobolinks is constructed, which indicates that an "average" bobolink may experience in autumn only a few weeks of day-lengths less than about 12¾ hours.

4. Beginning October 2, when captive bobolinks were experiencing natural day-lengths of about 12 hours 41 minutes, some were exposed to constant daily photoperiods of 12½ hours for four weeks, others to 12¾-hour photoperiods for five or six weeks, after which the photoperiod was increased to 14 hours. Testicular recrudescence occurred in all. It is concluded that photorefractoriness can be overcome in this species by only a few weeks of exposure to photoperiods which in length are comparable to those it encounters in transequatorial migration but which are longer than those which maintain refractoriness in such temperate zone forms as *Junco hyemalis* and *Zonotrichia albicollis.* The number of shorter days required for the termination of refractoriness seems to be of the same general order of magnitude as for *Junco* and *Zonotrichia.*

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DIGESTION, STORAGE, AND TRANSLOCATION OF NUTRIENTS IN THE PURPLE SEA URCHIN (STRONGYLOCENTROTUS PURPURATUS)[1]

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The internal transport of nutrients in echinoderms has been a matter of interest since 1809 when the French Institute offered a prize for a description of the "circulatory" system of asteroids, echinoids, and the holothuroids (Tiedemann, 1816). Since that time the anatomy of various echinoderms has been studied by numerous investigators, prominent among whom were Perrier, Hamann, and Cuénot. The accumulated knowledge of the Phylum Echinodermata was presented in a treatise by Hyman in 1955.

A survey of this literature indicates a general recognition of three fluid systems, *i.e.,* the perivisceral fluid, the water vascular system, and the haemal system. However, the roles of these systems in the translocation of food remain obscure.

The vessels of the water vascular system and the sinuses of the haemal system are very narrow and delicate. Sampling of the fluids that they contain is extremely difficult. Therefore, examination of transport in these systems has been limited to microscopic observations of the movement of objects within the vessels or sinuses, particles of injected dyes or the naturally present coelomocytes being the objects observed (Perrier, 1875; Kawamoto, 1927; see also Hyman, 1955). The perivisceral fluid, which bathes the internal organs, is relatively large in quantity and more accessible to sampling and subsequent examination. This fluid from representatives of the more conspicuous classes, namely the holothuroids, asteroids, and echinoids, has been subjected to physiological and biochemical analysis (Jacobsen and Millott, 1953; Lasker and Giese, 1954; Boolootian and Giese, 1958; Farmanfarmaian, 1959; see also Hyman, 1955). Some observations in these studies have resulted in the assignment of various possible functions to the variety of coelomocytes which are to be found in this fluid as well as in the tissues of the animals (Hyman, 1955; Stott, 1955; Boolootian and Giese, 1958).

The suggestion that these cells may be involved in nutrient transport is based on very little experimental evidence. Phagocytosis of foreign materials such as carbon, carmine, and fat particles and their deposition in certain tissues may or may not simulate aspects of natural nutrient transport. Since phagocytic cells are known to ingest such non-nourishing and inert particles as polystyrene latex spherules with subsequent migration *in vitro* (Sbarra and Karnovsky, 1959, 1960), no *a priori* significance may be attached to such processes when they are observed *in vivo.* Even studies involving the injection of materials such as sugars or iron saccharate (Lasker and Giese, 1954; Stott, 1955), though useful under special circumstances, cannot be regarded as good indicators of the natural process of transport following the digestion and absorption of food. The considerations mentioned above indicate the desirability of a systematic study of the translocation of nutrients following natural absorption. Knowledge of this phenomenon is prerequisite to the understanding of much of the biology of echinoderms. It has been possible to carry out such a study through the feeding of algae labelled with carbon-fourteen to sea urchins.

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MATERIALS AND METHODS

The purple sea urchin, *Strongylocentrotus purpuratus,* was chosen for these investigations. Animals with a test diameter of 4–7 cm. were starved for five to eight weeks prior to experimental feedings. The animals were maintained in well-aerated sea water at 15° C. in the laboratory.

The red alga, *Iridaea flaccidum,* was used as food in the experiments. (The name *Iridophycus* has been used to describe this genus also.) This material was chosen as food because it is abundant in the habitat of the urchin throughout the year; it constitutes one of the animal's natural foods as judged by the examination of gut contents in the field; and it is capable of maintaining urchins in good condition for over a year in laboratory aquaria when used as the sole source of food. Much of the biochemistry of this alga is known (Hassid, 1936; Bean *et al.,* 1953; Bean and Hassid, 1955).

Cut discs of the alga were labelled with C^{14} according to the method of Bean *et al.* (1953), using the gas-sealed apparatus, which they describe, as a photosynthesizing chamber. One to three grams of labelled alga could be prepared in this manner. Amounts of the alga less than one gram were labelled in sealed vials in a sea water solution of $NaHC^{14}O_3$ at pH 8. In either case the amount of $C^{14}O_2$ available to the alga was empirically adjusted to give $2–4 \times 10^4$ counts per minute/mg. wet weight of alga. The photosynthetic assimilation of $C^{14}O_2$ was allowed to proceed for 10 hours at about 18° C. The algal discs were sampled in several places to determine their specific activity. The discs were then drained and weighed carefully before being fed to the animals.

The animals were fed in sealed jars maintained at 15° C.; see Figure 1. These jars were provided with capillary air inlets which opened below the sea water and vacuum outlets from the gas phase. It was thus possible to draw off metabolic $C^{14}O_2$ into a $Ba(OH)_2$ trap and provide the animal with continuous aeration without contaminating the laboratory. The animals were never allowed more than five hours of feeding time. After the desired period of feeding, the remainder of the algal disc was removed and weighed as before.

All of the tissues were freshly sampled in such a manner as not to contaminate one another. This was achieved by careful dissection, the use of several sea water washes, and frequent changes of dissecting instruments. Duplicate samples were taken in all cases. When the final specific activity of duplicate samples differed by more than 10%, the samples were rejected. Rejection occurred most frequently when the gonads were ripe and spawning took place during sampling. Other sources of error responsible for large differences in duplicates were the difficulty of weighing fresh tissue to constant weight and contamination during dissection.

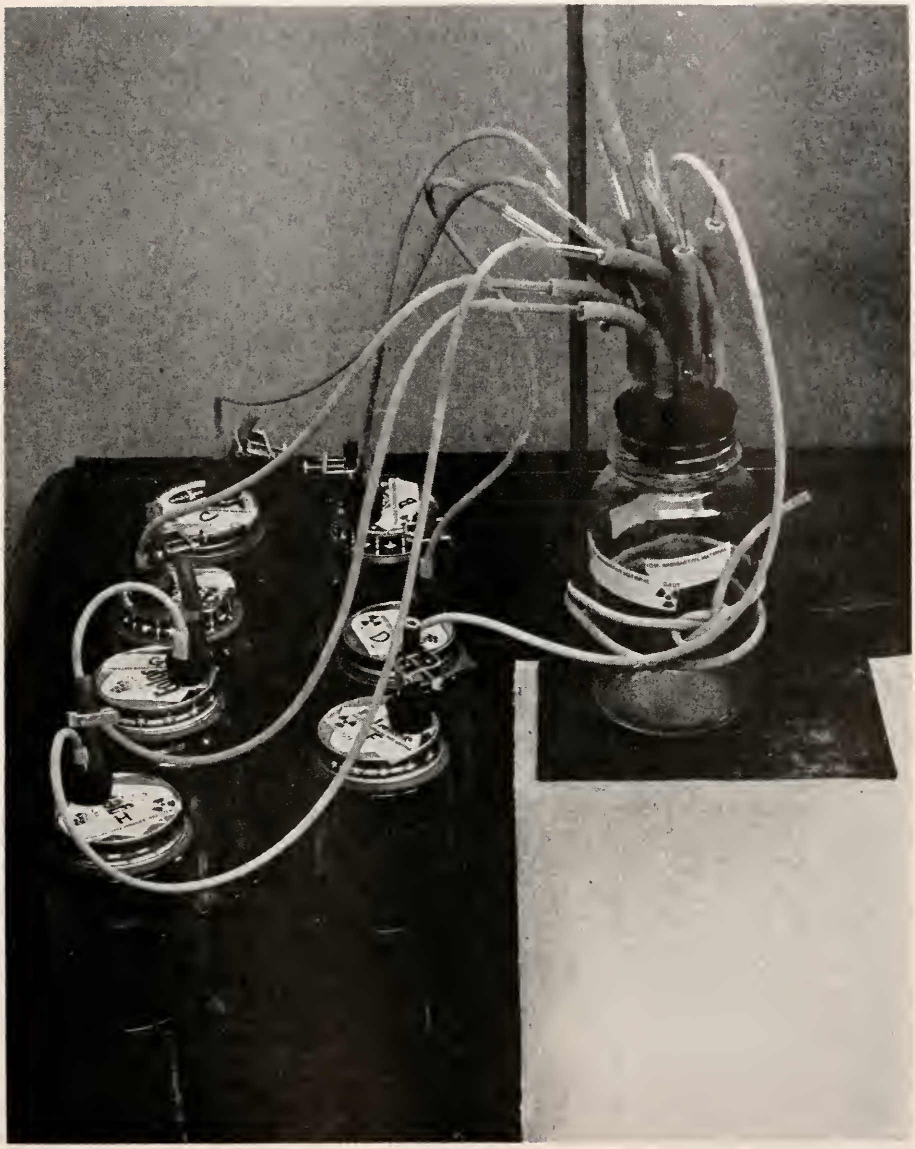


Figure 1. Arrangement of feeding jars in which sea urchins were fed radioactive algae.

The perivisceral fluid was sampled by a one-milliliter syringe through the peristomeal membrane and directly from the main coelom. A volume of 10% solution of ethylene diamine tetraacetic acid at pH 8.0 equal to the sample volume was used as anticoagulant. The cells and plasma were separated by centrifugation as desired.

Since the specific activities of various tissues were to be compared, it was necessary to use a homogeneous suspension or solution of the tissues. A high degree of homogeneity was attained by digesting samples of tissue in NaOH with the aid of 30% H_2O_2 and heat. Soft animal tissue samples of 10–20 mg. and 100–200 mg. samples of test wall were placed in graduated centrifuge tubes and covered to the 0.5-ml. mark with 1 *M* NaOH in sea water. Algal samples were covered with 10 *M* NaOH during the digestion and subsequently diluted with sea water in a manner calculated to keep the concentration of salts uniform. Time of digestion and the amount of H_2O_2 used were as needed. Unless otherwise stated, the activity of the perivisceral fluid was determined without digestion.

Samples of digests were placed on stainless steel planchets, dried, and counted in a Nuclear Chicago gas-flow counter, model D-47 with mica window, equipped with scaling unit model 161A, sample changer model c-110B, and printing timer model c-111B. All errors in the counting procedure were corrected by the methods of Calvin *et al.* (1949) and Kamen (1957). Particular attention was paid to errors in geometry and absorption because of the high salt content of the samples.

Paper chromatograms were analyzed by an Actigraph model c-100A combined with scaler model 1620A and recorder R1000.

RESULTS AND DISCUSSION

Digestion. The digestive tube of the purple sea urchin consists of a buccal pouch, a short pharynx, and an esophagus that enters the first convolution of the tube. This portion of the tube immediately below the esophagus will be referred to as the stomach. The stomach opens into the second convolution, referred to here as the intestine, and the latter terminates in a short rectum which opens to the outside via an anus. The stomach and intestine each have five festoons which will be designated one to five in sequence from mouth to anus.

The conversion of algal C^{14} into animal C^{14} was determined in the following manner: the specific activity of the alga pieces fed and the specific activity of the contents of the digestive tubes of animals sacrificed at various times were determined. These results are presented in Table I, and show that conversion efficiency, as is indicated by efficiency of digestion and absorption, is about 90% when the animal is fed a limited, 20 to 100 mg. wet weight, amount of *Iridaea*. Since the

TABLE I

Conversion efficiency of C^{14}-labelled substances from Iridaea *tissue into sea urchin tissue*

Days after start of feeding	C/M/mg. of dry digest of		% Activity incorporated	Nature of gut content
	Algae fed	Gut content		
1	526	38.6	93	Bite form No bags No bacterial enrichment
2	490	50.3	90	As above
3	555	36	93	Bag form Bacterial enrichment
4	641	53	91	Bag form High bacterial enrichment
9	294	33	89	As above

digestive efficiency remains nearly the same throughout the interval of one to nine days after a limited amount of food is ingested, the results suggest that digestion and absorption of at least the labelled portion of the food occurs primarily on the first day, and thereafter the remnants of food are on their way to defecation. Since the food has not passed the fourth festoon of the stomach by the second day, the esophagus, the stomach, or both would appear to be the main site of digestion in the animal. The algal material in the first four festoons of the stomach is essentially in bite form and free of bacterial enrichment. As is indicated in Table I, bacteria become conspicuous by the third day when the material has passed the fourth and fifth festoons of the stomach. At this point the material has been converted from the bite form to the bag form. These different kinds of gut contents are pictured in Figure 2. These observations suggest that the digestion of *Iridaea* in the stomach is not dependent on bacterial action and is more likely under the influence of digestive enzymes secreted by the sea urchin.

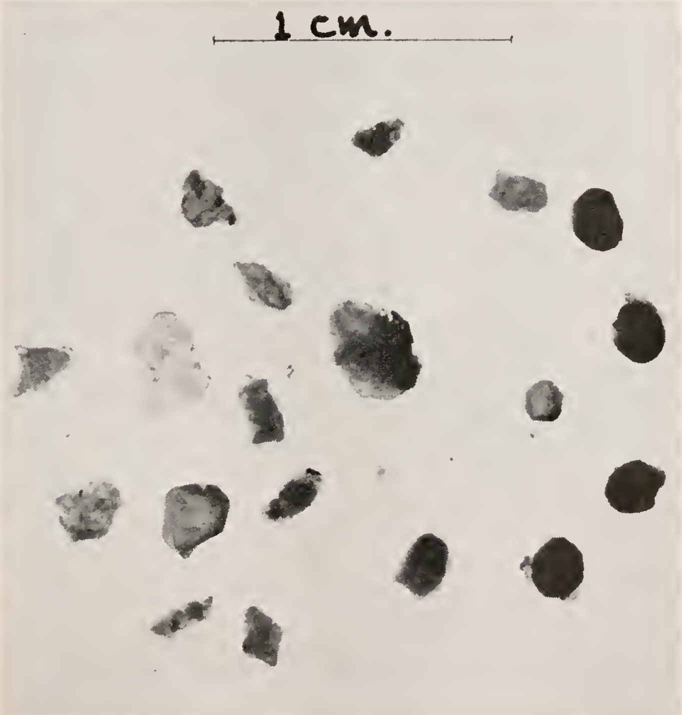


FIGURE 2. Pieces of *Iridaea* removed from various parts of the digestive tube. The transition from bite form from the stomach to bag form from the intestine is seen from left to right.

This suggestion was confirmed by examining the digestive ability of extracts of esophagus, stomach, and intestine. The tissues, washed free of gut contents, were homogenized in sea water and freed of large tissue fragments by centrifugation. Washed soaked agar and washed bite-sized pieces of *Iridaea* were used as substrates. Toluene was added to prevent bacterial growth. Reducing sugar was

TABLE II

*Reducing sugar liberated by tissue extracts**

	Agar	*Iridaea*
Extract of esophagus	76 μg†	60 μg
Extract of stomach	160	80
Extract of intestine	80	40
Pooled extract	240	80
No extract	56	40

* Twelve-hour incubation at 15° C.; 50 mg. of agar and *Iridaea* used as substrates. The amounts of tissue extract of esophagus, stomach, and intestine were 5, 71, and 78 μg protein. The pooled extract was a preparation containing equivalent amounts of each of the extracts. All values were corrected for the contribution of reducing material in the extracts.

† Values equivalent to μg glucose.

determined after incubation by the method of Park and Johnson (1949). Table II shows the results and conditions of incubation. It should be noted that some reducing material is solubilized from *Iridaea* and agar under the conditions of incubation. However, larger amounts appeared in solution after exposure to extracts of stomach or esophagus. The extract of intestine when tested by itself did not liberate additional reducing material from *Iridaea*. Whether or not it contributes to the material released by the extract pool remains to be determined. Both the stomach and the esophagus appear to possess digestive enzymes. Since the amount of material in the extract of esophagus is only one-fifteenth the amount in the other two extracts, the results suggest that the esophagus may contain appreciable amounts of digestive enzymes. The presence of enzymes capable of hydrolyzing agar is definitely indicated.

Lasker and Giese (1954) reported the presence of enzymes in extracts prepared from the whole digestive tube of the purple sea urchin. Enzymes capable of digesting casein, starch, and iridophycin, a galactan prepared by Hassid from *Iridaea,* were detected. Eppley and Lasker (1959) have demonstrated alginase and algin depolymerase activity in the digestive tract of this animal. The failure of Lasker and Giese (1954) to detect agar-digesting enzymes may have been due to their use of agar warmed to 37–40° C. Since this temperature is more than 10 degrees above the lethal temperature of the animal, it may result in inactivation of the enzymes responsible for agar digestion.

While the Aristotle's lantern of the sea urchin is a magnificent masticatory apparatus that is capable of reducing the alga to small pieces before it reaches the esophagus, the final disintegration of algal structure by the subsequent action of digestive enzymes has not been observed. There is no question that the bacteria from the intestine of the sea urchin can attack algae, but their role in the digestion of that part of the food which is normally assimilated may not be significant. When *Iridaea* is made available to the sea urchin in plentiful quantities, the animal often feeds continuously and defecates rapidly. Under these conditions the feces are usually in bite form and without bacterial enrichment. The disintegration of algal structure observed in the distal festoons of the intestine when defecation is delayed may provide additional assimilable material. The uptake of such material by the animal would not have been observed in the experiments reported here since labelling of the structural elements of the algal cells would have to have been accomplished during their growth. However, the lack of participation of microorganisms in the digestion of the labelled portion of the alga is further indicated by studies in which bacterial action was inhibited by antibiotics. C^{14}-labelled algal material kept in strong solutions of streptomycin and penicillin prior to feeding gave the same digestive efficiency and no bacterial enrichment of the feces.

That the microorganisms observed in feces do not constitute forms unique to the urchin is suggested by the following observations: finely chopped *Iridaea* in sterile sea water was incubated for four days in the dark at 15° C. Similar preparations were inoculated with bacteria-enriched contents of the rectum. Both series yielded a grossly similar collection of bacteria and protozoa. These preliminary experiments suggest that the fauna and flora of the sea urchin gut may represent the symbionts of the ingested alga. Mastication and digestion by the sea urchin render the material more susceptible to attack by these microorganisms and results in the bacterial and protozoan enrichment so often observed in the intestine of the animal.

Storage of digested food. From studies on the reproductive physiology and biochemistry of the purple sea urchin, the site of food storage has become a point of controversy (Giese *et al.*, 1958). It was, therefore, desirable to determine the site of deposition of C^{14}-labelled compounds in the animal. The overall specific activity of the animal was calculated in the following manner:

$$\text{Specific Activity of animal} = \frac{(\text{total activity of ingested algal material})\ (\text{conversion efficiency})}{(\text{total wet weight of the animal in mg.})}$$

The specific activities of various tissues of the sea urchin were determined and expressed in relation to the overall activity of the animal, the latter being given a value of 1.00. These results are presented in Table III. The esophagus and the first festoon of the stomach appear to be the main sites of nutrient storage. The drop in activity of the walls of the digestive tract by the seventh day after feeding was not accompanied by a general shift of material to all other tissues. It is well

TABLE III

Specific activity of various tissues relative to the calculated overall specific activity of the animal in counts/minute/mg. wet weight

Days after feeding	1	2	3	7
Tissue				
Whole animal	1.0	1.0	1.0	1.0
Body wall				
interambulacral	0.1	0.3	0.1	0.3
ambulacral	0.3	0.5	0.2	0.5
Gonad	1.6	1.0	0.8	1.3
Esophagus	20.4	60.0	26.3	21.2
Stomach				
Festoon 1	37.5	86.0	47.0	27.3
Festoon 4	7.9	14.5	5.2	11.8
Intestine				
Festoon 1	6.2	15.5	3.5	10.5
Festoon 4	2.3	12.0	1.6	4.7

established that a starving sea urchin will resorb its gonads, but there is no indication that the gonads constitute the natural storage organ of these animals. A recent study of Giese (1961) indicates that lipid is the main reserve food of *Strongylocentrotus purpuratus, S. franciscanus,* and *Allocentrotus fragilis.* The lipid is stored in the wall of the intestinal tract and is observed to decrease in amount during starvation. In the asteroids the hepatic caeca have been shown to be the organs of storage (Farmanfarmaian *et al.,* 1958; Anderson, 1953). Anatomically these are diverticula of the digestive tract, and the festoons of the sea urchin digestive tract may be compared to them in function.

TRANSLOCATION OF NUTRIENTS

1. *The haemal system*

In general this system consists of poorly defined sinuses often filled with red coelomocytes. No movement of fluid within any part of the system has been observed even though a rhythmic beat may be seen in the outer sinus of the stomach and its collateral sinuses. In spite of careful attempts, the fluid could not be sampled without contamination. Therefore, the role of the haemal system in the transport of nutrients was assessed by indirect means. C^{14}-labelled material appears in the gonads even on the first day after feeding; see Table III. Each gonad

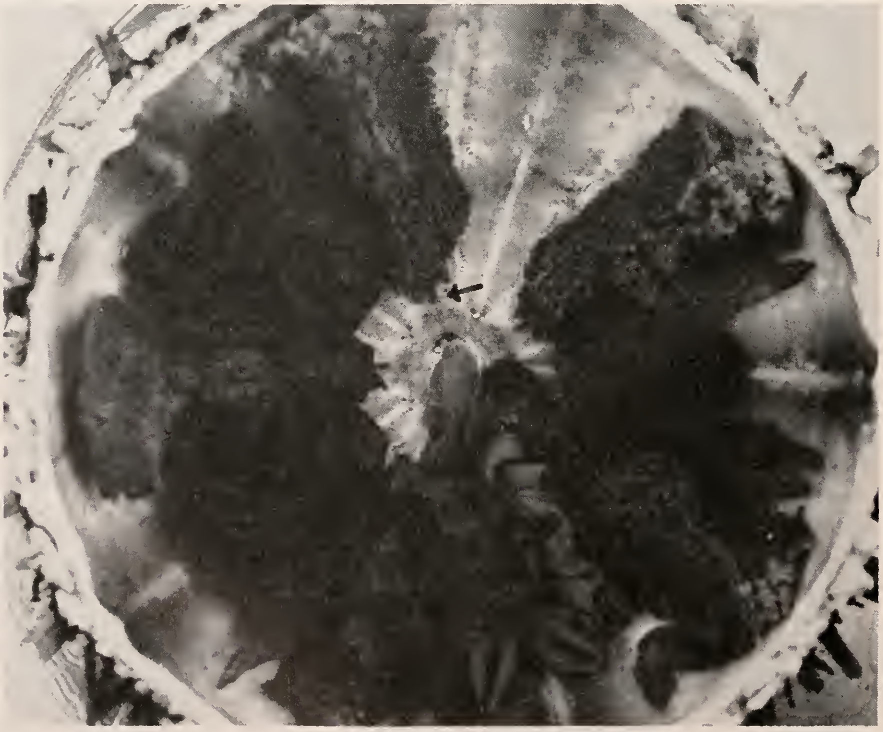


FIGURE 3. The aboral haemal ring and its short sinuses which penetrate the gonads. The arrow indicates one of the sinuses. Photograph is of a fresh specimen enlarged two times.

is penetrated by only one sinus from the aboral haemal ring. Therefore, it was possible to sever this sinus and compare the specific activity of a gonad thus isolated from the haemal system with its normal neighbor; see Figure 3. This delicate operation was accomplished by carefully drilling a half-millimeter hole just oral to the gonopore in the center line of the interambulacral region. Under a dissecting microscope equipped with a strong spotlight, the haemal sinus and the gonoduct of the gonad were seen just inside the test wall. These tubes could then be gently lifted up by means of a finely bent needle and severed by a microscalpel made from a piece of razor blade. The hole was closed with a fine wooden plug covered with Vaseline. The operation did no apparent harm to the animal nor altered its behavior in any observable manner. The cut tubes constricted and healed within 24 hours, and the connection between the aboral haemal ring and the gonad began to regenerate by about the tenth day after the operation.

Twenty-four hours after the operation, animals were fed C^{14}-labelled *Iridaea,* and the specific activity of the gonads was determined at intervals following feeding. The results are presented in Table IV. In all cases the isolated gonad and its neighbor contained essentially the same amount of activity, irrespective of sex or number of days after the start of feeding. The active materials, therefore, must have arrived via routes other than the haemal system.

TABLE IV

Comparison of the specific activity of normal gonad with neighboring gonad whose connection to the aboral haemal ring was experimentally severed

Days after feeding	Sex	Gonad gravidity	Animal wet weight in grams	C/M/mg. wet weight		
				Overall animal	Gonad experimental	Gonad control
1	♀	Ripe	31	160	275	253
2	♀	Ripe	32	24	26	25
3	♂	Ripe	25	230	177	181
7	♂	Ripe	25	42	54	55

These experiments were carried out on animals with ripe gonads. The possibility that the haemal system plays a special role in nutrient transport to the gonads during their period of buildup, *i.e.,* August through November (Giese *et al.,* 1958), must be examined by experimentation. However, data presented in a later section indicate the more general route of nutrient transport.

The function and microanatomy of the haemal system remain an enigma. This collection of sinuses does not appear to constitute a circuitous system, nor does it appear to have, functionally speaking, a point of origin or terminus. The enigma is no less striking when other classes of the phylum are considered (Hyman, 1955). The haemal system may be the vestige of a true transport system in the ontogeny or phylogeny of echinoderms.

2. *The water vascular system*

This system consists of a well-defined water vascular ring, the stone canal, and the five radial canals that penetrate the ambulacral regions through the auricles and thence send side branches to ampullae of the podia. Although coelomocytes may be observed to move within the lumen of the radial canals, there is no clear direction of flow. There is good evidence to support the suggestion that the canals of the water vascular system maintain the hydrostatic pressure required for the operation of the podia and their ampullae (Cuénot, 1948; Hyman, 1955).

Because of the difficulty encountered in attempts to sample the fluid of this system, an indirect method similar to that used for the haemal system was adopted.

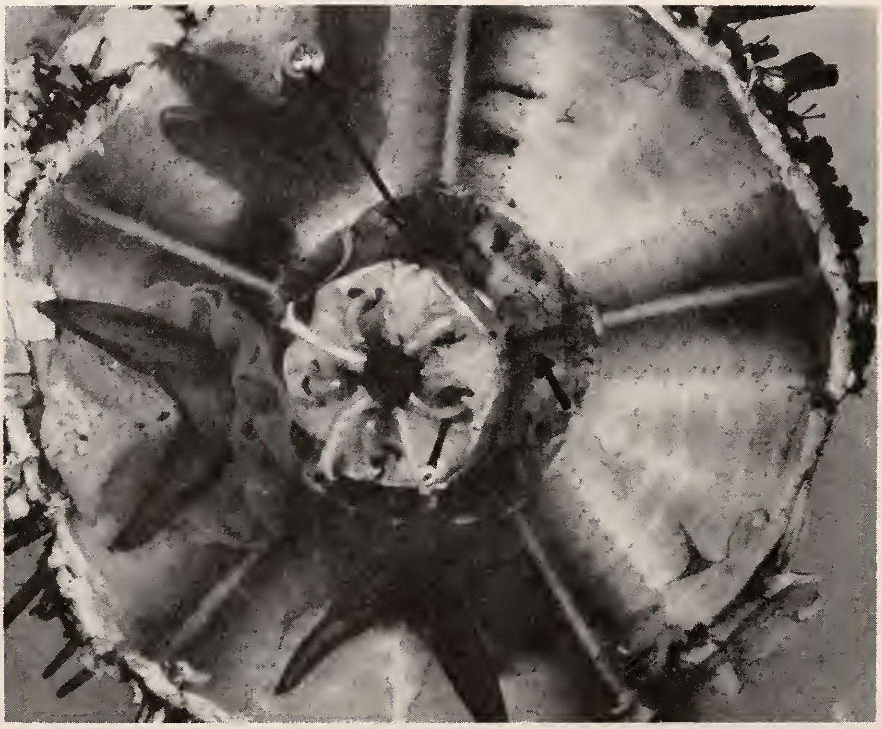


FIGURE 4. A view of the oral side of the animal from within. The lantern has been pushed to one side. The arrow points to the radial haemal sinus and the radial water canal just prior to their entrance into the orifice of the auricle. Photograph of fresh specimen twice enlarged.

The operation was considerably simpler because the radial haemal sinus and the radial water canal adhere firmly to the peristomeal membrane just prior to their entrance into the auricle. (See Fig. 4.) A 1-mm. incision through the soft peristomeal membrane across the center line of the ambulacral region exposed these tubes. The tubes were severed as described in the operation on the haemal system. Healing and regeneration are about the same as those described for the haemal system. Twenty-four hours after the operation, animals were fed C^{14}-labelled *Iridaea*, and the specific activity of the ambulacral areas determined in the usual manner. The results of these experiments are presented in Table V. No significant difference was noted between the ambulacral regions isolated from the radial haemal sinus and water canal and neighboring ambulacra which were left connected.

TABLE V

Comparison of the specific activity of normal ambulacrum with neighboring ambulacrum whose radial water canal and haemal sinus were experimentally severed at the auricle

Days after feeding	Animal wet weight in grams	C/M/mg. wet weight		
		Overall animal	Ambulacrum experimental	Ambulacrum control
1	31	160	53	55
2	32	24	14	13
3	29	28	13	12
7	25	42	22	22

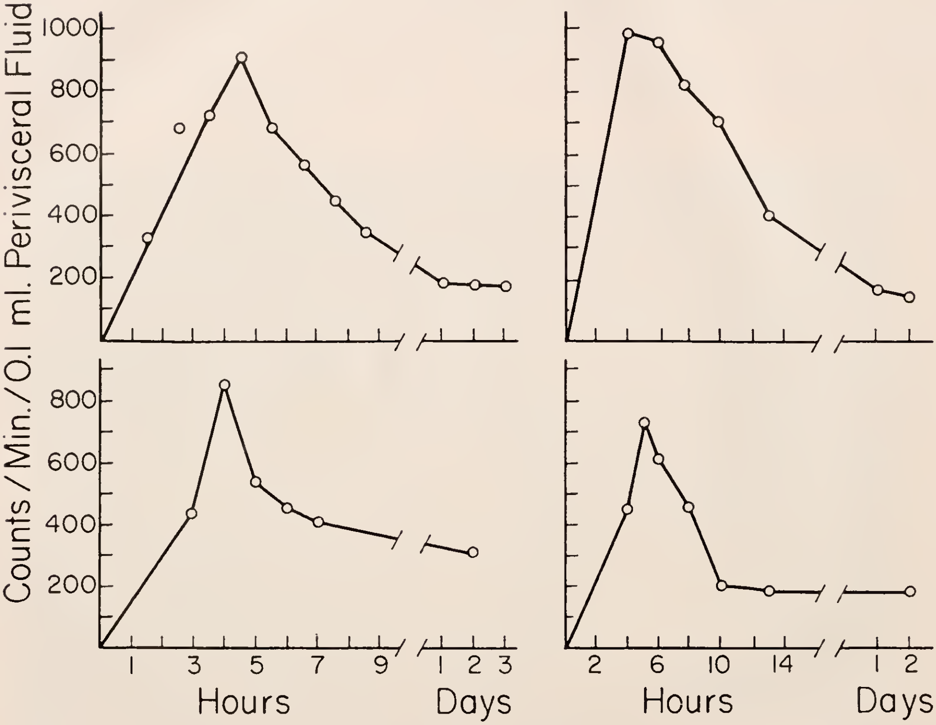
It seems reasonable to conclude that the water vascular system does not play a significant role in the transport of digested food under these circumstances. These experiments provide additional evidence for the lack of importance of the haemal system in this process.

3. *The perivisceral fluid*

The perivisceral fluid occupies the main coelomic chamber of the sea urchin and is kept in circulation by the cilia of the epithelial lining of the coelom. All the internal organs of the animal are bathed by this fluid. Numerous cells, the coelomocytes, of seven different kinds may be observed in this fluid (Boolootian and Giese, 1958). The plasma phase of this fluid contains low levels of nitrogenous compounds, carbohydrates, and possibly fats (Giese *et al.*, 1958; see also Hyman, 1955) and has essentially the same salt composition as sea water.

Since there are several milliliters of this fluid in the main coelom which may be tapped by a syringe via the soft peristomeal membrane, it was possible to sample this fluid directly and determine the level of activity at various intervals after the start of feeding. Figure 5 shows the results of a series of such experiments. The labelled substances reach a peak level within six hours and then decrease in concentration to a fairly constant level within the first 24 hours. Thereafter the level is generally maintained for at least nine days, the period during which samples were usually taken and examined. The general shape of the graphs in Figure 5 was independent of animal size and quantity of C^{14}-containing material fed.

In studies on the respiration of the purple sea urchin, it was demonstrated that the perivisceral fluid serves as a medium for respiratory gas exchange (Farmanfarmaian, 1959). It has also been demonstrated that the oxygen uptake of a starved urchin may increase by as much as 50% following feeding (unpublished data). The possibility that the peak levels depicted in Figure 5 are due to a gush of respiratory $C^{14}O_2$ was tested in the following way: duplicate sets of samples were taken from an animal. One set was acidified to pH 2 in order to convert any $C^{14}O_2$ to a volatile form. After drying, the activity in both sets was determined. Figure 6 shows the result of this experiment. The activity found in the perivisceral fluid is not due to respiratory $C^{14}O_2$, which must be present at any given time at a negligible level.



Rate of Appearance of C^{14}-labeled Substances in Perivisceral Fluid of Four Sea Urchins. Time after Start of Feedings

FIGURE 5.

The rise and fall in the activity of the perivisceral fluid suggested two hypotheses:

a. The transfer of nutrients from the digestive tube to the perivisceral fluid is controlled by some mechanism, *e.g.* neurosecretions. When a starved animal is fed, nutrients are rapidly mobilized, and a peak level of activity in the perivisceral fluid is observed. Within the first day after feeding, the tissues of the animal attain a state of relative sufficiency, and a feedback mechanism reduces and maintains the level of mobilized nutrients in a steady-state.

b. The peak level of activity in the perivisceral fluid is due to one or more labelled substances which are rapidly released and transferred to the perivisceral fluid by passive or active diffusion across the wall of the digestive tube. Since the quantity of algal material fed is restricted, the quantity of these diffusing substances is also limited. An initial peak is to be expected, and when the labelled substances are absorbed by the tissues, a steady-state is attained. In the steady-state, nutrients are mobilized from the reserves at the same rate as they are consumed by the tissues, or the steady-state is maintained due to some control mechanism.

These two hypotheses were tested by a series of experiments. Animals were

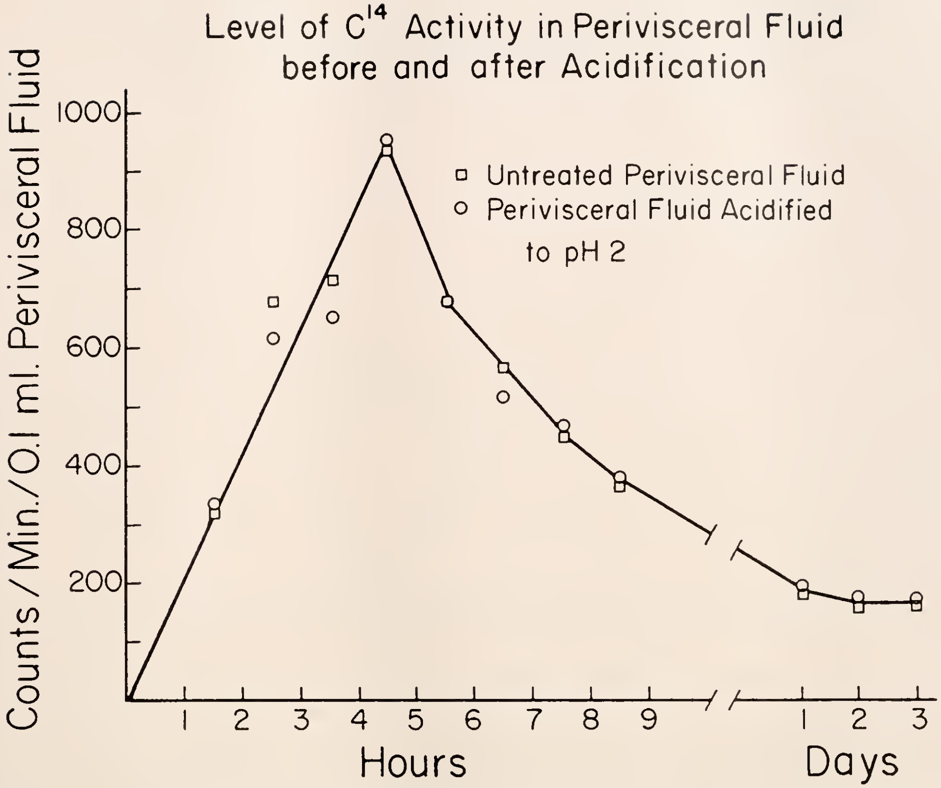


FIGURE 6.

fed labelled algae in the usual manner and the activity in the perivisceral fluid was determined. On the second day after establishment of the steady-state, the animals were fed again and the level of activity in the perivisceral fluid was again followed. Figure 7 shows the results of one of these experiments. If the first hypothesis were correct, a second peak would not have been expected. The first meal would have been expected to correct any nutrient deficiency of the tissues. Since a second peak was observed, the starvation preceding feeding was not responsible for the peak. The second hypothesis would appear to be more tenable, with the peak level representing material which rapidly diffuses from the gut into the perivisceral fluid. The possible involvement of a control mechanism in maintenance of the steady-state cannot be determined by these experiments and will require further study.

The role played by the coelomocytes in the transport of nutrients was determined by measuring the partition of activity between the plasma and cells of the perivisceral fluid at various intervals after the start of feeding. In order to achieve proper geometry for counting and to obtain comparable data, both the cells and the plasma were digested after separation and appropriate dilutions of the digests were pipetted onto the planchets for counting. Figure 8 presents the results and indicates that during the peak of the activity nearly all of the activity is in the plasma phase. Within one day from the start of feeding, as the steady-state is approached,

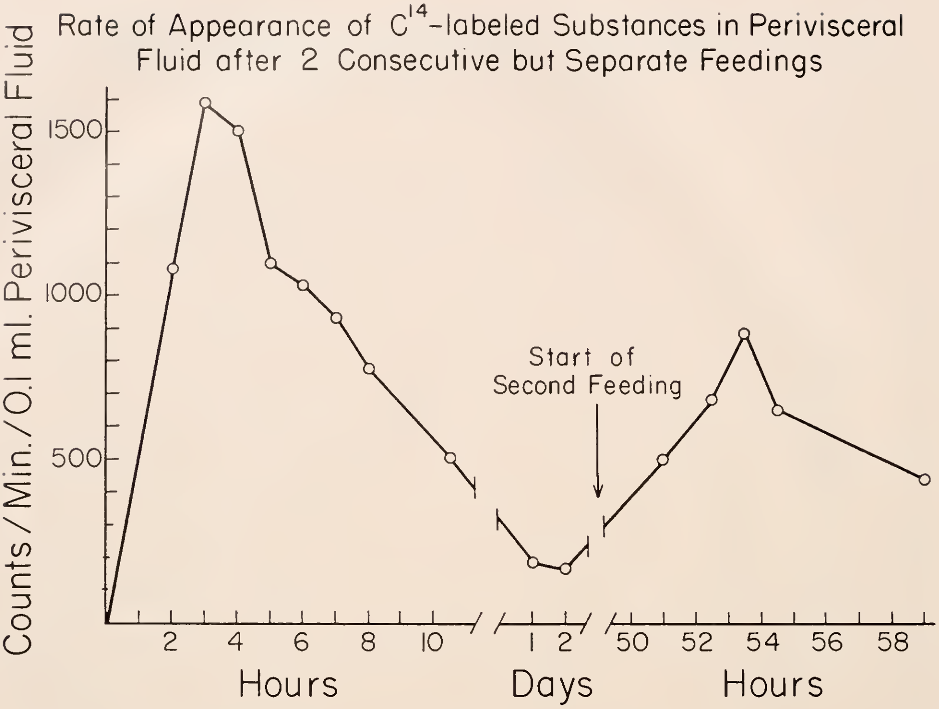


FIGURE 7.

the coelomocytes become the more heavily labelled phase. Table VI presents a set of similar results obtained with additional animals. At the peak level of activity more than 90% of the label is in the plasma. When the activity in the perivisceral fluid levels off, less than 50% of the activity remains in the plasma. Since coelomocytes, particularly the red eleocytes (Boolootian and Giese, 1958) may be observed in the wall of the digestive tube and other tissues, the presence of label in these cells adds support to the view that normal transport of nutrients from the site of reserves to other tissues may be partially achieved via the agency of coelomocytes. The above results do not, however, preclude the possibility that the labelled material in

TABLE VI

Distribution of C^{14}-labelled substances in the plasma and the coelomocytes of the perivisceral fluid of four sea urchins

Animal	Sampled at	Counts/Min/0.1 ml. of		% Activity in plasma
		Perivisceral fluid	Plasma	
A	Peak of activity	843	763	90.5
B	Peak of activity	1073	1070	99.5
C	"Leveled-off" activity	186	36	19.3
D	"Leveled-off" activity	250	116	46.5

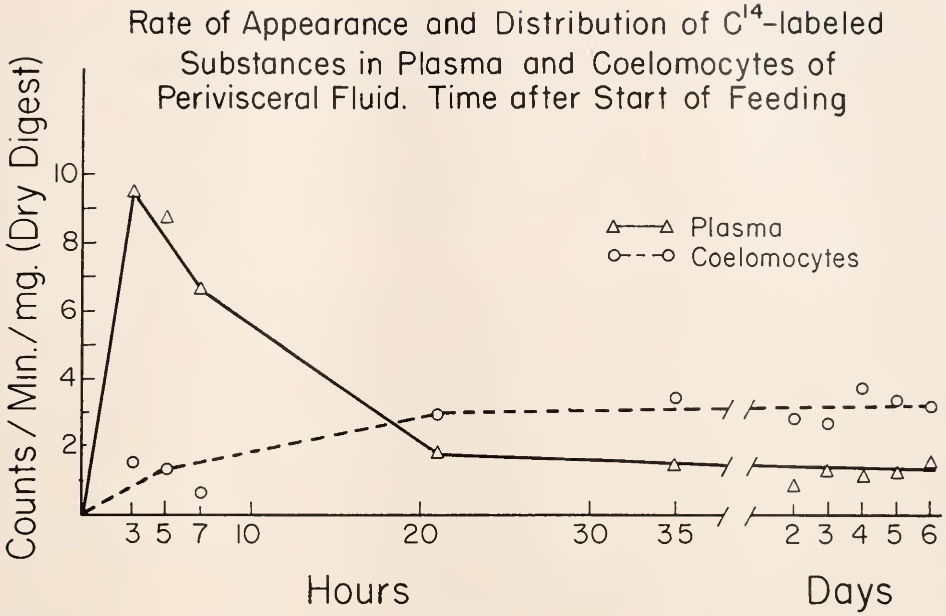


FIGURE 8.

these cells may be attributed to their own nutritional requirements; their appearance in the tissues of the animal may serve other functions.

Attempts were made to identify the labelled compounds found in the perivisceral fluid. Nearly 90% of the activity observed during the peak period was accounted for by one substance which was identified as galactose by its chromatographic behavior in three different solvent systems, conversion to mucic acid, and oxidation by galactose dehydrogenase (Block *et al.*, 1958; Doudoroff, personal communication). Free galactose is not a major constitutent of *Iridaea* tissue. The form of galactose most heavily labelled under the conditions of labelling used here is galactosylglycerol. None of this material could be detected in the perivisceral fluid. This galactoside is apparently hydrolysed by enzymes of the urchin gut.

After the peak level of activity is replaced by the establishment of a steady-state, the C^{14} is distributed among several compounds. Both carbohydrates and amino acids possess activity. Because the level of activity is very low, final identification of these compounds will require microtechniques which have not yet been attempted.

SUMMARY

1. In the purple sea urchin the digestion and absorption of the C^{14}-labelled constituents of the alga, *Iridaea*, occur mainly in the esophagus and adjacent festoons of the stomach.

2. The fauna and flora of the sea urchin gut do not appear to be involved in this digestive process.

3. The absorbed materials are stored mainly in the wall of the gut.

4. During absorption there is a diffusion of labelled material into the plasma of

the perivisceral fluid. A peak level is reached around the sixth hour after the start of feeding. Galactose accounts for 90% of this material and must have been liberated enzymatically from galactose-containing compounds such as galactosylglycerol.

5. The peak level of activity is replaced by a prolonged interval in which the level of activity is reduced but quite constant. The radioactivity is distributed over a variety of compounds including both amino acid and carbohydrates.

6. Translocation of nutrients is accomplished by the perivisceral fluid. No evidence for the participation of either the haemal or water vascular systems could be demonstrated.

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PHOTOMECHANICAL RESPONSES OF THE PROXIMAL PIGMENT IN PALAEMONETES AND ORCONECTES[1]

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Photomechanical adaptation of the crustacean compound eye in response to changes in illumination depends upon the behavior of the distal, proximal, and reflecting retinal pigments. The most recent review of the literature dealing with these pigmentary effectors was written by Kleinholz (1961). Much of the available information relates to the retinal pigments of the prawn *Palaemonetes vulgaris.* However, many of the investigators of retinal pigments in *Palaemonetes* and other crustaceans concerned themselves with the distal pigment only. Consequently, little is known about the physiology of the proximal and reflecting pigments. Parker (1896, 1897) was the first investigator to describe in a detailed manner the movements of the retinal pigments in *Palaemonetes.* Welsh (1930) later continued the study. He observed that migration of the distal pigment in light-adapting eyes was the result of shortening of the distal pigment cells. Sandeen and Brown (1952) found that the position maintained by this pigment in *Palaemonetes* is a function of the brightness of the visual field, not a true albedo response.

As far as endocrine studies are concerned, in 1936 Kleinholz reported that eyestalk extracts of *Palaemonetes,* when injected into dark-adapted prawns, caused light-adaptation of the distal and reflecting pigments but had no effect on the proximal pigment. More recently, evidence was obtained for a principle that dark-adapted the distal pigment. Brown, Fingerman and Hines (1952) observed that the response of the distal pigment to a light stimulus depended upon the light history of the prawns. The distal pigment of specimens kept in darkness overnight showed a much greater degree of light-adaptation in response to one minute of bright illumination than did this pigment in prawns preconditioned in dim light To explain this difference in response, the investigators postulated that a dark-adapting hormone was available to the prawns that had been exposed to the preconditioning low illumination but not to those that had been in darkness. Webb and Brown (1953) and Brown, Webb and Sandeen (1953) interpreted similar experiments in the same manner. Brown, Hines and Fingerman (1952) were able to increase the rate of dark-adaptation by injecting eyestalk extract into prawns at the time they were placed in the darkroom. Fingerman, Lowe and Sundararaj (1959) later were able to produce, by injection of eyestalk extracts, a dark-adaptational response in prawns maintained in light. Nothing further, however, has been done with the proximal and reflecting retinal pigments of *Palaemonetes.*

Information about the retinal pigments of the other crustacean used in this investigation, *Orconectes clypeatus,* is meager. The distal pigment of this crayfish is regulated by dark-adapting and light-adapting principles, just as in *Palaemonetes*

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(Fingerman, Mobberly and Sundararaj, 1959). The proximal and reflecting pigments of this organism have not been studied.

The primary objective of the present series of experiments was to obtain more information about migration of the proximal pigment in *Palaemonetes vulgaris,* especially the controlling mechanism. Kleinholz (1961) has suggested the possibility that this pigment is an independent effector. Among the experiments were some designed to determine (1) rates of migration of the proximal pigment under different experimental conditions, and (2) the character of the response of this pigment to a series of intensities of illumination. An experiment was also performed with *Orconectes,* by way of comparison with *Palaemonetes,* in order to determine whether the control mechanism could be the same for the proximal pigment of both species.

MATERIALS AND METHODS

Specimens of the prawn *Palaemonetes vulgaris* were obtained in the Woods Hole area weekly during the summer of 1960 through efforts of personnel from the Supply Department, Marine Biological Laboratory. We are extremely indebted to these individuals. The prawns were kept in large aquaria with running sea water. The crayfish, *Orconectes clypeatus,* were collected during the spring of 1961 in roadside ditches at Hickory, Louisiana, and were maintained in aquaria that contained aerated tap water. All of the experiments were performed at 22–24° C.

In order to determine accurately the position of the proximal retinal pigment, eyestalk sections, 20 μ thick, were prepared. In two of the experiments the position of the distal retinal pigment, also apparent in the sections, was of interest. The specimens were killed by immersion in boiling water for 10–15 seconds, thereby rapidly stopping additional migration of the retinal pigments. Both eyestalks were then removed from each specimen and placed in Bouin's solution until paraffin sections could be prepared. With the aid of a compound microscope, ocular micrometer, transmitted light, and reflected light, the positions of the pigments relative to the basement membrane could be precisely determined. Reflected light was used as an aid in distinguishing between the proximal and reflecting pigments. The difficulty of distinguishing between these pigments when transmitted light alone is used was noted by Kleinholz (1936). For the sagittal sections three measurements were made, just as was done by de Bruin and Crisp (1957) with the eyes of European crustaceans: (A) distance from outer corneal surface to distal edge of proximal pigment, (B) distance from outer corneal surface to distal edge of distal pigment, and (C) distance from outer corneal surface to basement membrane. The position of the basement membrane is constant. The ratio A/C was called the proximal pigment index; B/C, the distal pigment index. Use of ratios minimized the effects of size differences among the specimens.

EXPERIMENTS AND RESULTS

Palaemonetes vulgaris

Times required for light-adaptation and dark-adaptation of the proximal retinal pigment

The object of this set of experiments was to determine the length of time required for migration of the proximal retinal pigment of *Palaemonetes* from the light-adapted position to the dark-adapted one and back again. One group of prawns was placed in a darkroom for two hours and another group in white pans was exposed for two hours to an illumination of 560 ft. c. In a preliminary experiment this combination of background and intensity was sufficient to cause maximal light-adaptation of the proximal pigment. At the end of the two hours of preconditioning, 10 animals from each group were killed. The animals remaining in the darkroom were then exposed to 560 ft. c. while on a white background and the prawns that had been in light were put in the darkroom. At 15-minute intervals 10 animals from each group were preserved. The experiment was performed twice.

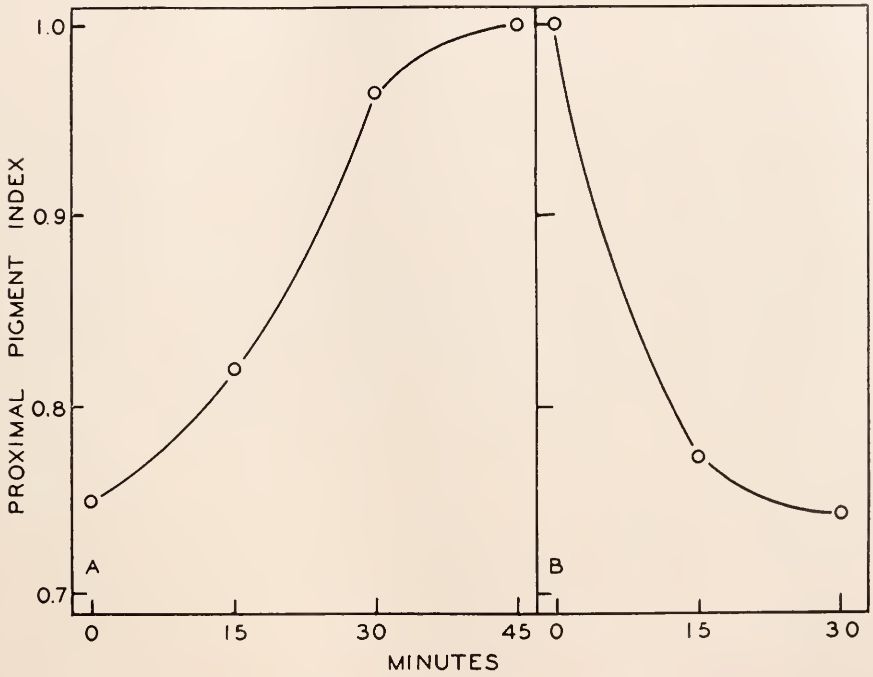

Figure 1. Relationships between the average proximal pigment index of *Palaemonetes* and time in minutes following transfer from light to darkness (A) and from darkness to light (B).

The means of the proximal pigment indexes are presented in Figure 1. Each point in the figure represents the average index of 20 eyestalks, each from a different prawn. Inspection of Figure 1 reveals that dark-adaptation required 45 minutes and light-adaptation 30 minutes. In a fully dark-adapted eye the proximal pigment index was 1.0; all of the proximal pigment had migrated proximal to the basement membrane. The proximal pigment index of a light-adapted eye was about 0.735. The mean maximal distance the proximal pigment had migrated distally in 38 fully light-adapted eyes was 92 μ. The mean distance from the outer corneal surface to the basement membrane in the same 38 eyestalks was 347 μ.

Relationship between light intensity and proximal pigment index of prawns on black and on white backgrounds

The objective of this experiment was to determine the manner in which the proximal pigment responds to different intensities of illumination and shades of background. To accomplish this purpose prawns in black and in white pans were exposed for two hours to one of a series of incident illuminations between 0.55 and 2230 ft. c. The latter intensity was sunlight. The other intensities were obtained by placing the containers of prawns at appropriate distances from the sources of illumination. Each intensity was measured with a General Electric photometer that had been calibrated at the Department of Physics, Newcomb College. Water in the pans exposed to the higher intensities was changed frequently to avoid heating of the prawns.

Inspection of the proximal pigment indexes revealed that at each illumination the pigment was in a more light-adapted position in prawns in white pans than in black ones. To learn whether this difference was a true background response depending upon the albedo, ratio of incident to reflected light, or simply a response to the intensity of reflected light, the fractions of the incident light reflected from the black and the white backgrounds had to be determined. The white background reflected one-half of the incident light but the black background reflected merely $\frac{1}{60}$ of the incident illumination. Then, on the basis of these data, the intensities of incident light were converted to intensities of reflected light. The mean proximal pigment indexes were subsequently plotted in Figure 2 against the logarithms of the reflected light intensities. Each point represents the mean of 12–20 indexes, the average number being 16.9. If the response depended on the albedo alone,

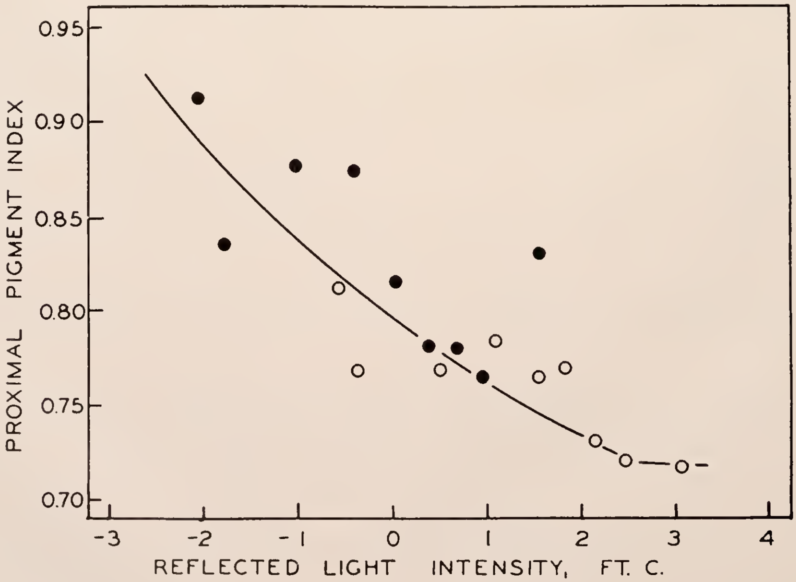

FIGURE 2. Relationship between the average proximal pigment index of *Palaemonetes* and the logarithm of the reflected light intensity. Circles, white background; dots, black background. See text for complete explanation.

then the data would have fallen along two distinct curves with no overlap of the indexes obtained from prawns on each of the two backgrounds. Such a situation is characteristic of chromatophore responses to background. However, inspection of Figure 2 revealed that the data fell more naturally along one curve. In the range of reflected light intensities between 0.55 and 12.5 ft. c. there was considerable overlap of the data. Maximal light-adaptation occurred at a reflected illumination of about 140 ft. c.

One must conclude from these data, therefore, that the position of the proximal pigment depends upon the intensity of illumination reflected from the surroundings rather than the albedo. Furthermore, the proximal pigment is functional over a wide range of light intensities and can be maintained in positions intermediate between the fully light-adapted and dark-adapted ones.

Relationships between (a) time in light and time required for re-dark-adaptation and (b) time in darkness and time required for re-light-adaptation

The object of this set of experiments was to learn whether the rates of light- and dark-adaptation of the proximal pigment can be altered by appropriate stimuli or whether these rates are independent of the light history of the animals. For use in the first set of experiments 16 white enameled pans, containing 10 animals each, were placed in the darkroom for two hours to assure maximal dark-adaptation of the proximal retinal pigment. At the end of the two hours, the prawns in one pan were killed. Subsequent observation of the eyestalks of the latter prawns revealed their average proximal retinal pigment index was 1.0, *i.e.* dark-adaptation of the proximal pigment had occurred. The remaining 15 pans were placed under an illumination of 250 ft. c. After five minutes in light, four of the pans were returned to the darkroom, four more were returned after 15 minutes in light, and another four after 45 minutes of illumination. With the return of each group of four pans to the darkroom, the prawns in a fifth pan were killed. The prawns in one of the pans from each of the three groups were preserved 10, 20, 40, and 60 minutes after their group had been placed in the darkroom the second time. This experiment was done twice and the data were qualitatively the same. The observed pigment indexes are presented in Figure 3A where each point represents the mean of 12–20 indexes, the average being 16.5. Zero time is the onset of the illumination period.

The proximal pigment of the prawns that had been in light for five minutes continued to light-adapt for at least 10 minutes after they had been put in the darkroom again. The indexes presented for the prawns at the end of the five minutes of illumintaion and after 10 minutes in darkness represent the means of 19 and 20 values, respectively. The subsequent rate of re-dark-adaptation was less than maximal. The proximal pigment of the prawns that had been in light 15 and 45 minutes slowly began to dark-adapt, but the rate increased with time. The proximal pigment of prawns that had been illuminated 45 minutes was more nearly dark-adapted after 60 minutes than it was in either the 5- or 15-minute groups after each had been in the darkroom for an hour.

The reciprocal experiment was performed next. Sixteen white enameled pans, each containing 10 prawns, were exposed to an illumination of 250 ft. c. for two hours. At the end of this period of conditioning, prawns from one pan were killed

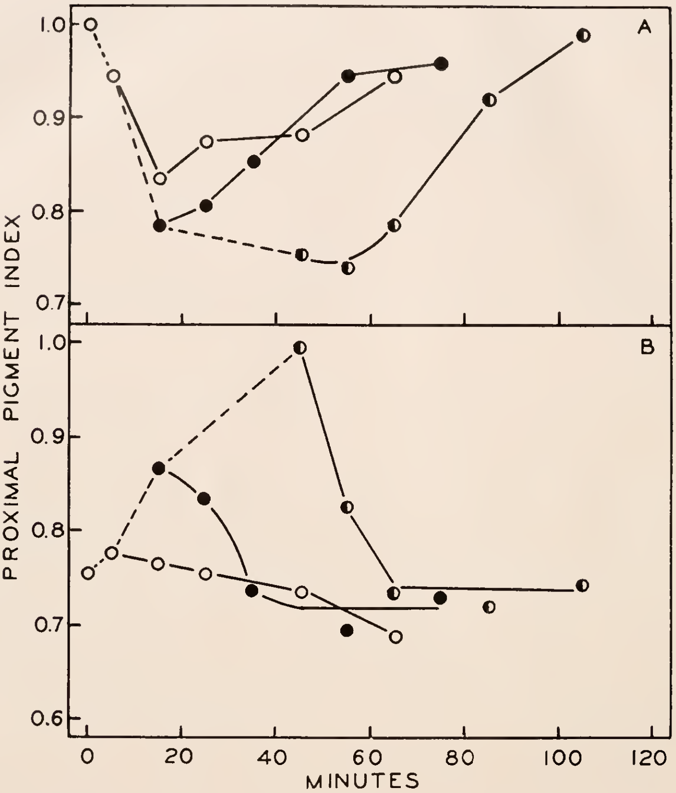

FIGURE 3. Relationships between mean proximal pigment index of *Palaemonetes* and time in minutes. A, dark-adapted prawns illuminated and then returned to the darkroom; B, light-adapted prawns put in the darkroom and then returned to light. Circles, 5 minutes; dots, 15 minutes; half-filled circles, 45 minutes in light (A) or dark (B). The dashed lines connect the initial indexes of each group of prawns.

and the eyestalks preserved. The remaining 15 pans were placed in the darkroom. After five minutes in the dark, four of the pans were returned to an illumination of 250 ft. c., four more were returned after 15 minutes, and another four after 45 minutes. With the return to light of each group of four pans, the animals in a fifth pan were killed. The position of the pigment 10, 20, 40, and 60 minutes following the return of each group to light, was determined by fixing the eyestalks of the animals from one pan at the appropriate intervals. The experiment was repeated. The averaged data are presented in Figure 3B where the mean number of indexes represented by each point ranges from 14 to 20, the average being 17.6. The rate of re-light-adaptation was a direct function of the time spent in darkness; the longer animals were kept in the dark (after having been previously light-adapted), the more rapid was the rate of re-light-adaptation.

Influence of light history on the response of the retinal pigments to a high intensity of illumination for one minute

The aim of this set of experiments was to determine the character of the response of both the distal and proximal pigments to one minute of high intensity illumination in prawns kept overnight (a) in darkness and (b) in the stock aquarium. The prawns that had been in the stock aquarium overnight had been exposed to the gradually increasing illumination of dawn. At 5:00 A.M. on the morning the experiment was performed, 30 animals from the stock aquarium were divided equally among three white enamelled pans and exposed for one minute to an illumination of 250 ft. c. After this bright stimulus the animals in one pan were killed

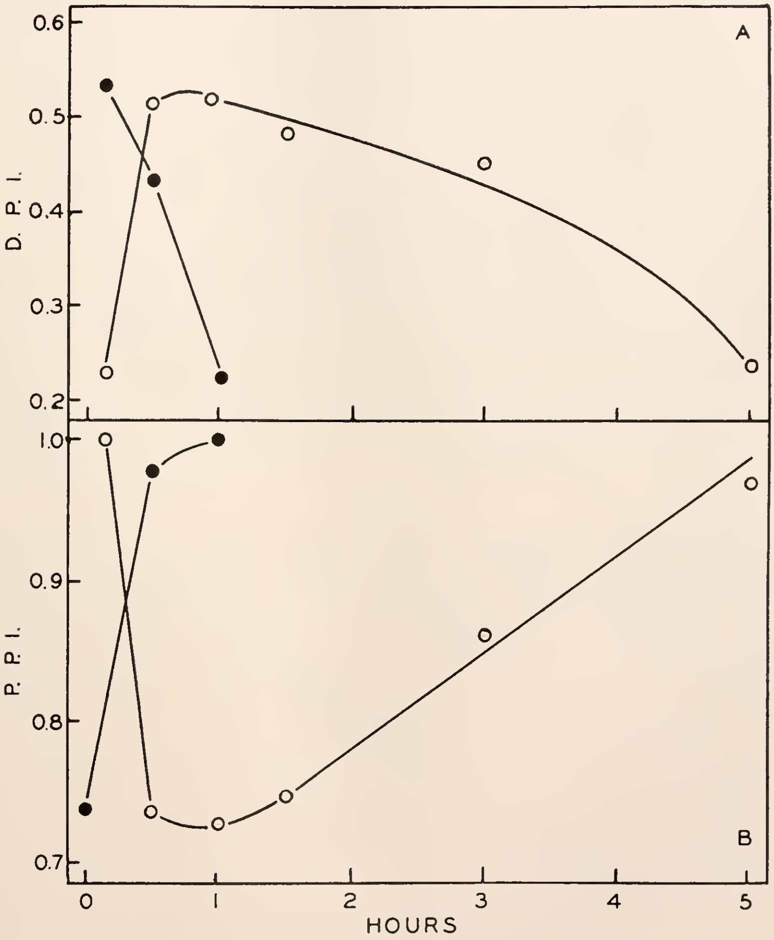

FIGURE 4. Relationships between time in hours and (A) average distal pigment index (D. P. I.) and (B) average proximal pigment index (P. P. I) of *Palaemonetes* kept in darkness overnight (circles) and of prawns kept overnight in the stock aquarium where they were exposed to dawn (dots). At 5 A.M. both groups were exposed to an illumination of 250 ft. c. for one minute.

and their eyes preserved. The remaining pans in the meanwhile were placed in the darkroom. After 30 minutes in darkness, the animals from one pan were killed. The remaining 10 prawns were killed after 60 minutes. Also at 5 A.M., six white enameled pans, containing 10 prawns each, were taken from the darkroom after having been there since 5 P.M. the previous evening and were exposed for one minute to the same 250 ft. c. The animals in one pan were killed immediately. The remaining five pans were returned to the darkroom. Prawns in one of the pans in the darkroom were killed 30, 60, 90, 180, and 300 minutes after the return to the darkroom. This experiment was performed twice.

After the eyestalks were sectioned, the indexes of the distal and proximal pigments were determined. The averaged data were used in the preparation of Figure

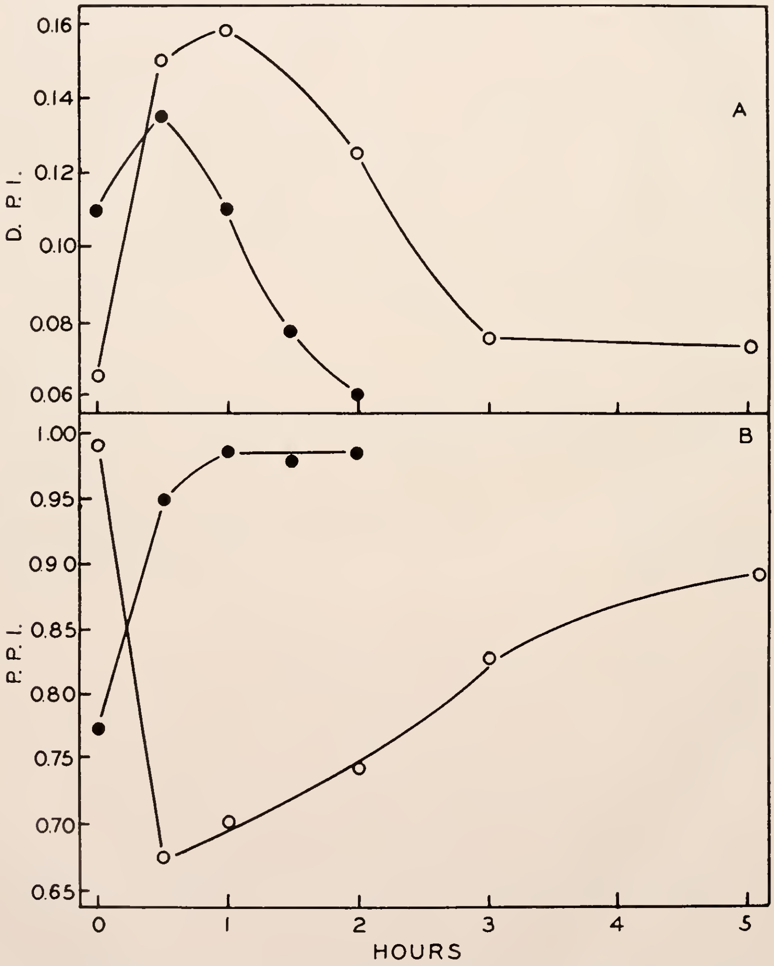

FIGURE 5. Relationship between time in hours and (A) average distal pigment index (D. P. I.) and (B) average proximal pigment index (P. P. I.) of *Orconectes* kept in darkness overnight (circles) and of prawns illuminated overnight under one ft. c. (dots). At 5 A.M. both groups were exposed to an illumination of 250 ft. c. for one minute.

4 where each point represents 13–20 indexes, the mean being 17.4. It is apparent from inspection of this figure that re-dark-adaptation of the distal and proximal pigments occurred at a very slow rate in the prawns kept in darkness overnight; one minute of bright light produced a light-adaptational response of both pigments that required about five hours to subside. On the other hand, dark-adaptation of both pigments occurred at the maximal rate in the *Palaemonetes* that had been exposed to dawn.

Orconectes clypeatus

Influence of light-history on the response of the distal and proximal pigments to a light stimulus of one minute duration

This experiment, performed twice, was essentially the same as the one described immediately above with the single change in protocol having been that one group of the crayfish was exposed to an illumination of one ft. c. from 5 P.M. to 5 A.M. rather than the gradually increasing illumination associated with dawn. The objective of this experiment was to learn whether under similar experimental circumstances the proximal and distal pigments of *Orconectes* would respond in the same fashion as the pigments of *Palaemonetes*. If such a situation was observed, then presumably the controlling mechanism would be similar in both organisms. The averaged data for the distal and proximal pigments are presented in Figure 5 where each point represents the mean index for 20 eyestalks. The mean distance from the outer corneal surface to the basement membrane in 40 eyestalks was 318 μ. Inspection of the figure reveals that the proximal and distal pigments of the crayfish that had been in darkness overnight responded with a light-adaptational response that lasted for at least three hours, whereas the pigments of the *Orconectes* illuminated all night dark-adapted more rapidly. The distal pigment of the latter group showed a light-adaptational response in darkness whereas the proximal pigment dark-adapted in one hour.

Discussion

The experiments described above provide some basic information concerning the proximal retinal pigment of *Orconectes* and especially *Palaemonetes*. Parker (1896, 1897) had reported that dark-adaptation of the proximal pigment of *Palaemonetes* required 45–60 minutes; light-adaptation, 30–45 minutes. Dark- and light-adaptation of the proximal pigment in the *Palaemonetes* used in the present investigation required 45 and 30 minutes, respectively (Fig. 1). The latter times were the same as the minimal values presented by Parker. Repetition of Parker's experiment seemed justified in view of the lack of agreement some investigators have noted with Parker's (1896, 1897) data on migration of the distal pigment of *Palaemonetes*. Parker observed that light- and dark-adaptation of the distal pigment required 90–105 and 105–120 minutes, respectively. Welsh (1930) later reported that light-adaptation occurred in 40–50 minutes and dark-adaptation in 80–90 minutes when observed in living specimens, but when the pigment was observed in fixed and sectioned eyestalks the corresponding times were 50–60 and 90–120 minutes. On the other hand, Sandeen and Brown (1952) stated that light-adaptation of the distal pigment in *Palaemonetes* required about 95 minutes and dark-adaptation 60 minutes. De Bruin and Crisp (1957) found that light-adaptation of the proximal pigment in the mysid *Praunus flexuosus* and the prawns *Palaemon* *serratus* and *Pandalus montagui* required 4, 4, and 4–6 minutes, respectively, under an illumination of 1.1 ft. c. Light-adaptation of the distal pigment in the respective organisms required 20, 90, and 40 minutes. As in *Palaemonetes,* more time was required for light-adaptation of the distal pigment in *Palaemon, Praunus,* and *Pandalus* than for the proximal pigment.

The conclusion that the position of the proximal pigment in *Palaemonetes* is a function of the reflected light intensity and not the albedo is the same as that of Sandeen and Brown (1952) who studied the distal pigment of the same species. The black background merely serves to decrease the intensity of illumination reflected onto the eyes. This observation is not too surprising when one considers that specimens of *Palaemonetes,* when swimming, extend their eyestalks at right angles to the body. Because of this swimming posture (1) shielding of the ommatidia by the body is minimal and (2) most of the ommatidia are stimulated by light reflected directly from the bottom and walls of the container and indirectly by internal reflections at the water-air interface. Very few of the facets appear to receive stimulation directly from the light source, especially when the illumination is a point source.

The results of the experiments concerned with alterations in the rate of dark-adaptation (Figs. 3A, 4, 5) indicate that the proximal pigments of *Palaemonetes* and *Orconectes* are not independent effectors, responding directly to illumination, but rather are under endocrine control. The proximal pigment of prawns that had been in light for only five minutes continued to light-adapt after they were put in the darkroom again (Fig. 3A). This response is strong indication of the release of a light-adapting principle in response to the dark-to-light change.

The striking difference in behavior of the proximal pigment observed when the effect of one minute of bright light was determined with animals maintained in darkness overnight and prawns exposed to the increasing illumination of dawn (Fig. 4, *Palaemonetes*) or to a low intensity of illumination all night (Fig. 5, *Orconectes*) can also be readily interpreted in terms of endocrine regulation of this pigment. The results presented for the distal pigment in Figures 4 and 5 are similar to findings of Brown, Fingerman and Hines (1952) and Webb and Brown (1953) for the distal pigment of *Palaemonetes.* The data for the distal pigment of *Palaemonetes* were included to illustrate the similarity in behavior of this pigment and the proximal pigment. One possible explanation of the behavior of the proximal pigment in *Palaemonetes* and *Orconectes* kept in darkness overnight is that a large quantity of light-adapting hormone was released as a result of the one minute of bright light. This hormone presumably accumulated in darkness. In the prawns exposed to illumination prior to 5 A.M. a faster rate of dark-adaptation was observed. Presumably, the light-adapting principle did not accumulate in illuminated prawns. Brown, Fingerman and Hines (1952) and Fingerman and Mobberly (1960) found that the distal pigment light-adapting hormone accumulated in specimens kept in darkness. A second possible explanation for the rapid rate of dark-adaptation of the second group of prawns is that the low intensity of illumination was responsible for providing the animals with the ability to dark-adapt rapidly by stimulating production and storage of dark-adapting hormone. However, the experiments do show that the light history of the animals greatly influenced their ability to light- and dark-adapt. This observation makes it extremely difficult to accept the hypothesis that the proximal pigment of *Palaemonetes* is an independent effector. More likely, the proximal pigment is controlled by at least one blood-borne principle, and conceivably light-adapting and dark-adapting hormones may both be involved.

Summary and Conclusions

1. Light-adaptation of the proximal retinal pigment of *Palaemonetes* required 30 minutes; dark-adaptation, 45 minutes.
2. The proximal pigment of *Palaemonetes* more closely approached the light-adapted position when the prawns were on a white background than on a black background under the same incident light intensity. The black background functioned merely to decrease the brightness of the visual field.
3. The proximal pigment in specimens of *Palaemonetes* and *Orconectes* kept overnight in darkness showed a greater light-adaptational response after exposure to a high-intensity light stimulus than specimens that had been under a low intensity of illumination prior to the bright light.
4. Evidence was presented in support of the hypothesis that light-adaptation of the proximal retinal pigment of *Palaemonetes,* following a dark-to-light change, is due to discharge of light-adapting hormone.
5. The data were discussed in relation to the findings of other investigators.

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PROFLAVIN AND ITS INFLUENCE ON CLEAVAGE AND DEVELOPMENT [1]

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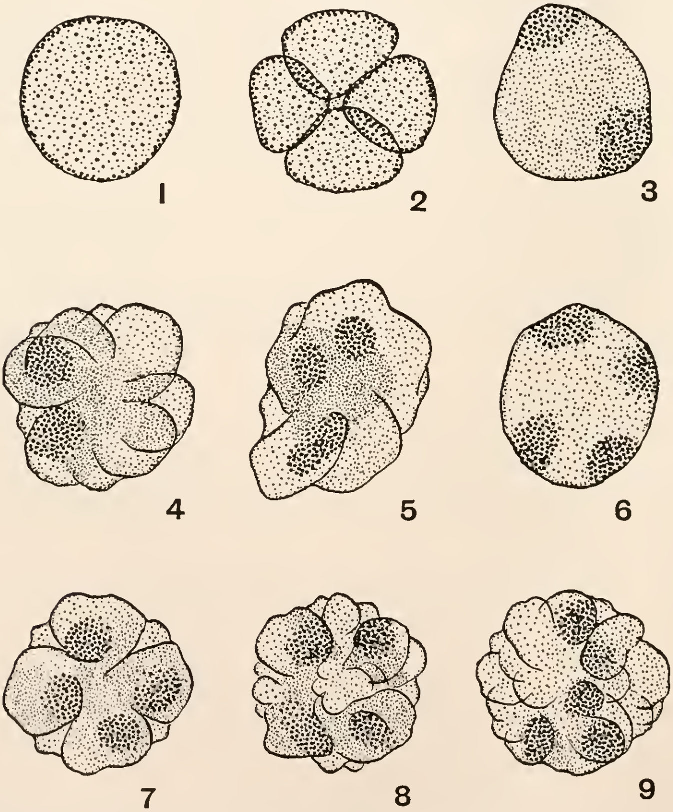
Proflavin is one of the acridine dyes and has been shown (Lerman, 1961) to combine directly with nucleic acids; the combined product becomes highly photosensitive. It is a very effective mutagen. One would suppose that a study of the influence of proflavin on the mitotic figure of the *Arbacia* egg might, from the nature



FIGURES 1, 2. Normal cells, showing regular distribution of pigment granules.
FIGURES 3 to 9. Pigment granules in segregated masses.

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[1] It has long been the policy of The Biological Bulletin not to accept very short papers or brief notes. Because of the distinguished contributions of Dr. Ethel Browne Harvey to the Marine Biological Laboratory, an exception is being made in this case.—Editor.

of proflavin, give interesting results. Light is necessary for the action of proflavin; in the light as well as in the dark, cleavage goes on normally, whether proflavin is present or not. In the light as well as in the dark, proflavin was found to have no influence in producing cleavage without the mitotic figure, as I had thought possible (1960). So far I have found no influence of proflavin on the mitotic figure. It does, however, cause a delay in cleavage of one or two hours, and often inhibits cleavage completely.

The amount of proflavin to be used to give the best results has been found to be 10 micrograms per cc. of sea water. This should be made up frequently and kept in the refrigerator. If not subjected for too long a period (over 50 minutes?) or in too great a strength the action is reversible. The strength should be not greater than 10 micrograms per cc. of sea water.

The most striking effect of the proflavin is to cause a concentration of the red pigment granules into two or more large masses of granules. In the normal egg, the pigment is scattered throughout the cells as small pigment granules. These remain, in eggs treated with proflavin, as segregated masses, becoming more numerous with time, without any tendency to combine. Even when the *unfertilized* eggs are treated with proflavin, cleavage (after fertilization) is delayed and abnormalities occur, and the pigment is concentrated in masses in the cleavage cells. The accompanying drawings, made by Eve Chambers, Woods Hole, Mass., show the pigment granules in the normal unfertilized egg scattered throughout the cytoplasm (Figs. 1, 2) and later (Figs. 3 to 9) aggregated in masses in the somewhat irregular cleavage cells. Viscosity is greatly increased by proflavin, as found also by Lerman (1961).

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RATE OF PHOSPHORUS UPTAKE BY PHAEODACTYLUM TRICORNUTUM [1]

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Research concerning phosphorus relations in unicellular algae has been active and profitable in several fields—oceanography, limnology, ecology, plant physiology, and biochemistry (Kamen and Spiegelman, 1948; Rice, 1953; Ketchum, 1954; Arnon, 1956; Bradley, 1957; Krauss, 1958; Steele, 1959; and many others). Two problems that have not yet been adequately studied are the uptake of phosphorus by given species of algae under controlled conditions, and the influence of algal populations on the recycling rates of phosphorus in whole communities. These problems are closely related, and ecologists recognize that the recycling rate is as fundamental a parameter of a community as the absolute abundance of algae and of nutrient phosphorus (Ketchum *et al.,* 1958). As an approach to the problem of measuring community recycling rates, we measured the rate at which *Phaeodactylum tricornutum* Bohlin (previously called *Nitzschia closterium* forma *minutissima* Allen and Nelson) (Lewin, 1958) accumulated both the abundant phosphate in fresh medium and the scant phosphate of P^{32} carrier.

The authors wish to express their appreciation to Mr. N. Corwin for performing many of the chemical phosphorus analyses; to Dr. V. T. Bowen for use of his radioisotope laboratory facilities and for criticism of the manuscript; and to Dr. R. R. L. Guillard for criticism of the manuscript.

METHODS

Chemical analysis. The particulate (cellular) fraction was separated from the dissolved phosphorus fractions by filtering samples of algal culture through either a $BaSO_4$ precipitate deposited on a sintered-glass filter funnel, or a millipore membrane filter, pore size 0.8 μ, previously washed by drawing 100 ml. of 10% HCl and several copious rinses of distilled water through it. Inorganic phosphate in samples of filtrate was determined by the Deniges-Atkins colorimetric method as described by Wooster and Rakestraw (1951). The color was measured in a photoelectric colorimeter (Ford, 1950) using a red filter (Corning 2408) and a 9- or 29-cm. light path. Total dissolved phosphorus in other samples of filtrate and cellular phosphorus caught upon the $BaSO_4$ precipitate or membrane filter were determined by the Harvey (1948) method as modified by Ketchum *et al.* (1955) using a 29-cm. path length in the photometer. Phosphorus concentration was calculated by use of appropriate factors obtained by calibration with standard solutions.

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Strickland and Parsons (1960) reported the limit of sensitivity of the phosphate method to be about 0.08 μg. at P/1 (μg. at P/1 = μM); the method for total phosphorus has about the same limit.

Radioisotope analysis. The dissolved P^{32} was separated from the cellular P^{32} fraction by filtering a 10-ml. portion through a membrane filter. The cells on the filter were not washed because such treatment leaches phosphorus from cells (Rice, 1953). To estimate the P^{32} held by sorption on the filter, we made "sorption" blanks by placing two or three membrane filter discs together on the filter holder so that the cells were caught on the upper disc and only the filtrate passed through the lower discs. The filter disc with the cells, the sorption discs, and 1-ml. aliquots of the filtrate were dried on separate planchets for counting with an end-window G-M detector connected to a scaler. Corrections were not necessary for self-absorption, for geometry of the detector, nor for coincidence losses, but corrections were made for decay when applicable.

The amount of vacuum applied to draw the water through the filter was one source of error in determining the P^{32} distribution between water and cells. To evaluate this, part of a culture of *Phaeodactylum* was freed of living cells by centrifuging it and then adding a drop of formalin to the centrifugate to kill any residual cells. To this, and to another portion of the culture containing living cells, equal amounts of P^{32} were added. Both were placed in the dark for more than an hour to permit equilibration with the P^{32}. When the centrifugate without living cells was drawn through three consecutive filters, only about 3% of the radioactivity was found on the filters, and most of that on the upper one (#1, Table I). Higher vacuum resulted in somewhat lower amounts of activity on the filters, presumably because less interstitial water remained. When the culture of living cells was filtered, practically all of the P^{32} was in the cells and was therefore retained by the top filter. The amount of activity in the filtrate increased with increased vacuum, and the activity on the sorption filters (#2 and 3) was quite variable (Table I). Although the amount of P^{32} on the sorption filters from the culture was much lower than that from the cell-free centrifugate, it was large compared to the P^{32} in the filtrate. The increasing P^{32} in the filtrate with intensified suction suggested physical damage to the cells, with labeled particles and soluble fractions being caught on the

TABLE I

Radioactivity detected on membrane filters and in the filtrate at different degrees of vacuum expressed as the differential between atmospheric and flask pressures. Values are counts per minute (cpm) in 10 ml. of centrifugate or culture

	Vacuum (mm. Hg):	50	100	200	250	360	510	660
Centrifugate:	Filter #1	460			450			330
	2	140			110			110
	3	140			120			90
	Filtrate	20,700			19,600			20,700
Culture:	Filter #1	18,200	18,200	18,400		18,000	18,200	18,600
	2	4.7	9.5	6.2		6.5	3.1	2.4
	3	4.4	5.3	2.8		1.4	2.0	1.9
	Filtrate	4.0	8.0	5.0		19	11	25

lower millipore filters and passing through with the filtrate. The measured activity in the filtrate of experiments I to V (Table II) is probably an overestimate because the importance of gentle vacuum was not then known; the suction used was usually in the range 250–500 mm. Hg. In experiments VI to X the pressure differential was kept below 50 mm. Hg. Because of the release of some phosphorus from the cells during filtration, the radioactivity on the sorption blank was added to the activity of the cells.

TABLE II

Comparison of concentration of phosphate in culture medium as measured chemically with that calculated from the minimum concentration of P^{32} during the experiment.

$$\text{Calculated } PO_4 = \frac{\text{minimum filtrate } P^{32}}{\text{total } P^{32}} \times \text{Total } P$$

Experiment	Chemical analysis (μM)		P^{32} (cpm/10 ml.)		Calculated filtrate PO_4 (μM)
	Filtrate* PO_4	Total P, cellular and dissolved	Filtrate minimum	Total, cellular and dissolved	
I	.11	1.77	10	4,180	.0042
II	.15	2.42	7	7,730	.0022
III	.16	4.06	4	7,890	.0021
IV	.16	4.79	8	7,720	.0050
V	.11	8.43	110	7,620	.12
VI	(.41)	1.37	32	47,500	.00093
VII	(.36)	1.62	23	52,000	.00072
VIII	(.50)	2.19	45	51,500	.0019
IX	(.50)	3.25	83	199,000	.0014
X	(.41)	5.63	80	203,000	.0022

* The results in parentheses are not considered dependable because of unusually large blanks.

Measurement of phosphate concentration by isotope partition. The partition of P^{32} between cells and medium was used to measure the dissolved phosphate concentration when it was near or below the limit of sensitivity of chemical analysis. Assuming that algae absorb $P^{32}O_4$ and $P^{31}O_4$ to the same degree, and that they do not release labeled phosphorus compounds other than orthophosphate, at equilibrium the ratio dissolved P^{32}/total P^{32} should equal the ratio dissolved PO_4/total P. Therefore, dissolved PO_4 = (total P) (dissolved P^{32})/(total P^{32}). This calculation undoubtedly overestimates the real concentration for the following reasons: (1) equilibrium, and hence the minimum dissolved P^{32}, may not have been reached when the experiment was terminated; (2) even gentle suction may damage some cells, releasing labeled phosphorus; and (3) the assumption of negligible release of labeled soluble organic phosphorus compounds during even short experiments may be false. The dissolved PO_4 calculated from P^{32} in nine cultures equilibrated in the dark was less than 4% of the value obtained by chemical analysis (Table II). Only in experiment V do the two values agree closely.

These results suggest that in media equilibrated with P-deficient algae the major part of the PO_4 measured chemically was liberated from acid-labile phosphorus compounds in the filtrate by the acid-molybdate reagent during the chemical analysis, and that only a small fraction was present as free phosphate ions in the whole culture. The values in Table I show that phosphate from cells damaged during filtration would have been too low to detect chemically. We took the values derived from isotope partition to be the real phosphate concentrations and used them for further calculations.

EXPERIMENTAL RESULTS

Uptake from phosphate-rich media. The net uptake of phosphorus by a pure culture of *Phaeodactylum* was studied by analytical chemical techniques. A culture was grown in constant light in "f-medium" (Guillard and Ryther, 1961) (Table III) until cell multiplication had reduced the phosphate concentration in the medium and the phosphorus content of the cells to a low level (4.2×10^{-15} mole/cell). Ten-ml. portions of this culture were added to each of four flasks containing 480 ml. of "f-medium" with various phosphate additions, giving 44×10^7 cells/liter in each flask. The dissolved phosphate concentrations were 8, 16, 32, and 80 μM (flasks A, B, C, and D, respectively). The flasks were illuminated (400 foot-candles) at 20° C., and 10-ml. samples were filtered periodically to measure dissolved and particulate phosphorus. The sensitivity and standard deviations were about 1 μM in samples containing low concentrations of phosphorus because of a 20-fold dilution during the analyses; at the highest concentrations the standard deviations were as much as 5 μM because dilutions as great as 100-fold were necessary. The cells were counted periodically using a Spencer Bright-Line counting chamber.

TABLE III

Composition of "f-medium" (Guillard and Ryther, 1961)

$NaNO_3$	150 mg. (1.76 mM)
$NaH_2PO_4 \cdot H_2O$	10 mg. (72.5 μM)
Fe sequestrene*	10 mg. (1.3 mg. Fe or 23.3 μM)
$Na_2SiO_3 \cdot 9H_2O$	30 mg. (3 mg. Si or 106 μM)
Vitamins:	
Thiamin·HCl	0.2 mg.
Biotin	1.0 μg
B_{12}	1.0 μg
Trace metals:	
$CuSO_4 \cdot 5H_2O$	0.0196 mg. (0.005 mg. Cu or 0.079 μM)
$ZnSO_4 \cdot 7H_2O$	0.044 mg. (0.01 mg. Zn or 0.153 μM)
$CoCl_2 \cdot 6H_2O$	0.020 mg. (0.005 mg. Co or 0.085 μM)
$MnCl_2 \cdot 4H_2O$	0.360 mg. (0.1 mg. Mn or 1.83 μM)
$Na_2MoO_4 \cdot 2H_2O$	0.013 mg. (0.005 mg. Mo or 0.052 μM)
Sea water	To one liter

* Sodium iron salt of ethylene dinitrilo tetraacetic acid (EDTA). Ferric chloride and EDTA or the sodium salt of EDTA can be mixed to give the same amounts of iron and the chelator; adjust pH to about 4.5. Ferric sequestrene is made by Geigy Industrial Chemicals, Saw Mill River Road, Ardsley, New York.

The time course curves of phosphate uptake by phosphorus-poor *Phaeodactylum* (Fig. 1A) are similar in shape, but the period of accumulation was much extended at high initial phosphate concentrations. Whereas it took 6 days before the phosphate was depleted in flask D, it was depleted in two hours or less in flask A. In

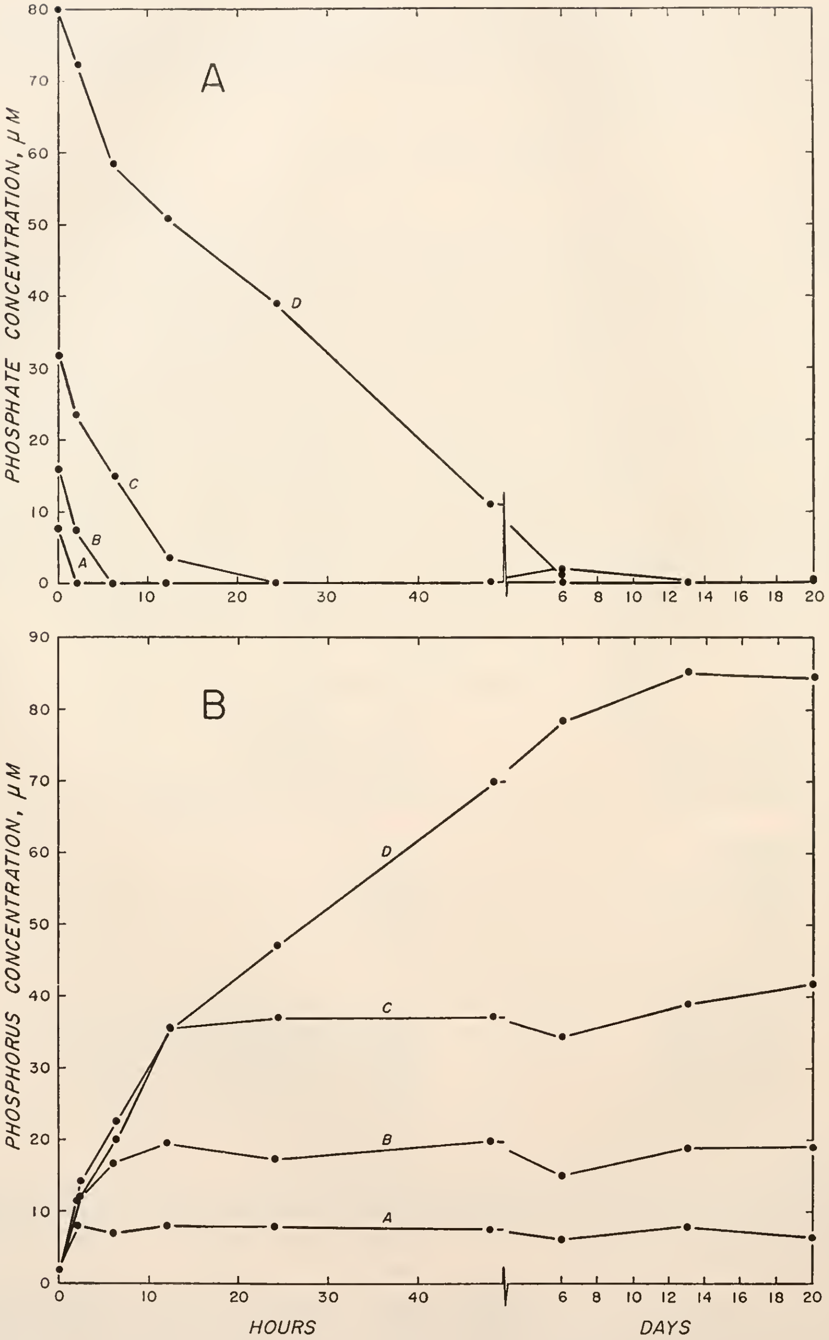

FIGURE 1. The changes of dissolved phosphate in the media (1A) and of intracellular phosphorus (1B) when *Phaeodactylum* was grown in media of different initial phosphate content. The initial cell count was 44×10^7 cells/liter and the initial phosphate concentrations were 8, 16, 32 and 80 μM, for A, B, C, D, respectively.

all cultures the phosphate was not significantly different from zero after 13 days. The phosphorus content of the cellular fraction (Fig. 1B) was the inverse of the phosphate content. The apparent increase in total phosphorus concentration at the end of the experiment is consistent with the usual rate of evaporation of water from media. The difference between total dissolved phosphorus and dissolved phosphate, frequently referred to as dissolved organic phosphorus, was never a significant amount in A or B, but reached a maximum of about 8 μM in flasks C and D at 6 and 12 hours, respectively. In all cultures it was not significantly different from zero after the sixth day.

The quantity of phosphorus per cell, shown in Figure 2, reached a maximum in two to 12 hours, the peak occurring later in the flasks with higher initial phosphate

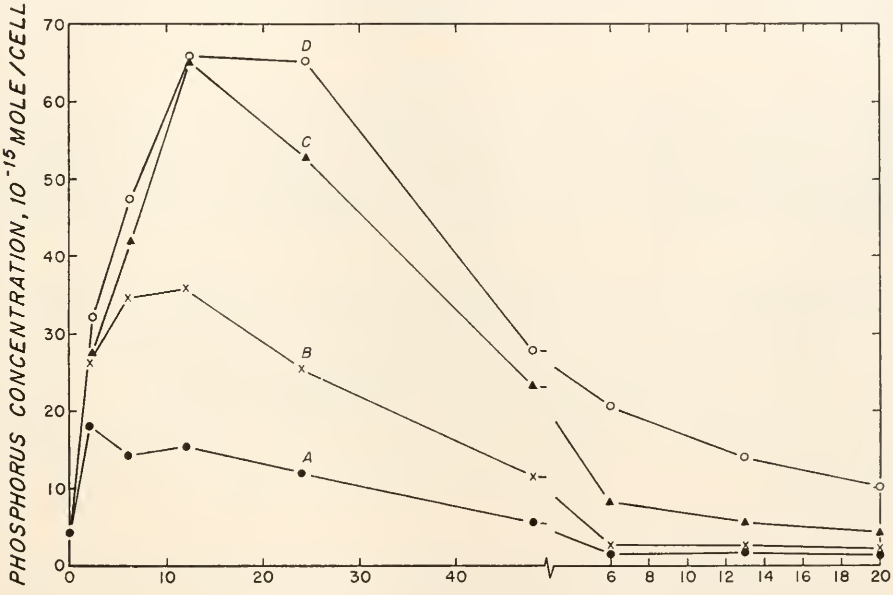

FIGURE 2. Phosphorus concentration of *Phaeodactylum* as a function of time after inoculation into media with varying initial phosphate concentrations as in Figure 1.

concentrations. The maximum concentration per cell in flasks A, B, and C was nearly a function of the initial phosphate concentration in the medium because these cultures were able to remove almost all of the phosphate before appreciable cell multiplication occurred. The number of cells in flask D, however, nearly doubled in the first 24 hours, at which time about half of the phosphate still remained in solution. These cells reached their peak concentration in 12 to 24 hours. The later decreases in phosphate per cell resulted from continuing cell multiplication.

The increase from 4 to 66 × 10^{-15} mole/cell (flasks C and D), a factor of 16, in 12 hours is remarkable. Part of the increase per cell may be attributed to cell enlargement prior to division, but much of the increase must represent a greater concentration of phosphorus in the protoplasm. Negligible uptake by adsorption was shown by an experiment described below. As growth of the culture continued, the intracellular concentration declined until, at about 2×10^{-15} mole/cell in flask A, *Phaeodactylum* became phosphorus-deficient and stopped multiplying. In the other flasks cell division continued at a slow rate up to 20 days. The final cell counts varied from 400 to 960×10^7 cells/liter.

The rates of phosphate uptake by *Phaeodactylum* were neither linear, owing to removal of a constant quantity of phosphate per unit time, nor exponential, owing to removal of a constant proportion per unit time, but were intermediate. The linear, or mean, rates are simply the uptake of phosphate per cell divided by the duration of each sampling interval. The exponential rate at the beginning of each interval was calculated by

$$k = \frac{\ln P_t - \ln P_0}{n \cdot t} \tag{1}$$

and

$$v = k \cdot P_0 \tag{2}$$

where P_0 and P_t are the phosphate concentrations in solution at the beginning and end of a sampling interval, t is time in minutes, n is the cell density (number per volume), k is the velocity constant, and v is the instantaneous initial uptake rate. Both k and v are negative. The exponential and linear rates are shown in Table IV.

Table IV

Rates of phosphate uptake by Phaeodactylum in the light (400 fc) in media of high phosphate concentration. The first column for each experiment is the instantaneous initial rate calculated assuming an expontential rate of phosphate uptake; the second column is the mean rate assuming a linear uptake during the interval. Values are 10^{-17} mole/cell·minute

	A		B		C		D	
Initial PO_4 (μM):	8		16		32		80	
Approx. time (hours)	Initial	Mean	Initial	Mean	Initial	Mean	Initial	Mean
0–2	(32)*	13	24	17	15	13	13	12
2–6	—	—	(13)*	6	10	8	14	12
6–12	—	—	—	—	12	6	5	4
12–24	—	—	—	—	(1)*	1	3	3

* Undependable because of extremely low final phosphate concentration.

The agreement between flasks A to D during each time interval demonstrates that in all four experiments the rate of supply of PO_4 to the cells did not limit the uptake rates as long as PO_4 was chemically detectable. The rates were thus more dependent upon the physiological condition of the cells than upon the PO_4 concentrations. The decreasing rates in C and D as the cells became glutted (Fig. 2) after about 6 hours are also apparent.

The uptake of P^{32} by living, phosphorus-poor *Phaeodactylum* was compared to that of an equally dense suspension of chloroform-killed cells in darkness at 22° C. Separation by filtration showed that during the first hour the living cells accumulated 93% of the added P^{32} whereas the chloroform-killed cells took up only 3%.

The living cells continued their uptake, but the dead cells changed little during the next two hours. On the basis of this experiment and others in which cells were killed by heat, toluene, or chloroform, it was concluded that physical sorption by the cells was not an important factor in the rapid uptake of phosphorus by algal cultures.

Ketchum (1939a) showed *Phaeodactylum tricornutum* to be capable of accumulating 25 × 10^{-15} mole/cell in 48 hours of darkness. This value is an order of magnitude lower than the values in Table IV. The highest net uptake rates reported for *Phaeodactylum* by Ketchum (1939b), when the cells were added to medium containing about 1 μM phosphorus and illuminated, were about 20% of the rate of experiment A (Table IV) during the first two hours. His lower rates may be the result of the lower phosphate concentrations, the lower experimental temperature, the longer sampling interval, and perhaps a different physiological condition of the cells.

Uptake from phosphate-poor media. The rates of uptake at low phosphate concentrations were determined by adding P^{32} as phosphate to equilibrated cultures and measuring the rate of change of radioactivity in the medium. Sea water medium was prepared with five different concentrations of phosphorus and one-tenth the concentration of other nutrients of "f-medium." The phosphorus and iron were added aseptically after autoclaving to minimize formation of precipitates because such precipitates rapidly remove P^{32} from the water. Silicon was omitted from the medium since it does not limit growth of *Phaeodactylum.* Equal inocula from a phosphorus-poor culture were added to 500 ml. of each of the five media. The flasks were placed in darkness at 20° C. for 24 hours. Cell counts were made for each flask. Triplicate 100-ml. samples were then removed, and the cells were separated by filtration for phosphate, total dissolved phosphorus, and cellular phosphorus determinations. Practically all of the phosphorus at this time was in the cells (Table V). Radioactive phosphate was added to the remainder of each culture and they were kept in darkness at 21–25° C.

Table V

Phosphorus concentrations, cell counts, and uptake rates of Phaeodactylum in the dark in media of low phosphate concentrations

Experiment	Phosphorus concentration			Total P^{32} (10^5 cpm/l)	Cell count (10^7 cells/l)	Uptake rate (10^{-17} mole/cell min.)
	as PO_4 in filtrate (μM)	as cells in culture (μM)	within cells (10^{-15} mole/cell)			
I	0.054	1.8	6.1	4.4	29	3.0
II	0.052	2.4	10	8.2	23	2.7
III	0.052	4.1	18	8.5	23	2.2
IV	0.055	4.8	19	7.8	25	3.0
V	0.17	8.3	34	8.4	24	0.97
VI	0.011	1.2	8.2	48	15	1.3
VII	0.011	1.5	9.4	52	16	1.1
VIII	0.012	2.0	12	52	16	1.1
IX	0.041	3.1	19	200	16	3.2
X	0.042	5.5	34	210	16	3.0

Ten-ml. portions of the culture were filtered periodically during the next three hours and the P^{32} content of both the cells and the water was determined. The uptake of P^{32} by the cells proceeded smoothly and rapidly, the time course curves being logarithmic. It was from these data that we concluded that the chemical method of analysis overestimated the low concentrations of dissolved phosphate in the medium. The final equilibrium value in these cultures is given in the last column of Table II. This value plus the amount of carrier phosphate added with the P^{32} gives the original phosphate concentration listed in Table V. This assumes that the cells had reached equilibrium after the 24-hour dark period and reached the same equilibrium after assimilating the radio- and carrier phosphate during the three-hour experimental period. The amount of carrier phosphate added (0.01–0.05 μM) was large compared to the dissolved phosphate but small compared to the total phosphorus in the culture.

The rate of phosphate uptake was computed by a formula derived from (1) and (2):

$$v = \frac{\ln P_t^* - \ln P_0^*}{n \cdot t} \cdot P_0 \tag{3}$$

in which P_0^* and P_t^* are the P^{32} concentrations in solution at the beginning and end of the interval and P_0 is the initial total dissolved phosphate content. The other symbols are as previously defined. The rates computed for the first 7–9 minutes of the experiment are given in Table V which also gives the total activity and the cell count for each culture.

The rates of uptake from the low phosphate concentrations by these cells are an order of magnitude less than those in Table IV where the cells were lower in phosphorus content and the dissolved phosphate concentrations were much greater. The rates at low concentrations showed no definite trend related to either the phosphate concentration or to the phosphorus content of the cell.

Discussion

The two experiments described were designed to measure phosphate uptake under contrasting conditions. In the first experiment (Table IV) phosphorus-deficient cells were transferred to a medium containing large amounts of phosphate relative to the amount in the cells. The different amounts formed a source of supply for periods ranging from about two hours to six days. The initial assimilation, when growth and cell division were negligible, increased the phosphorus content of the cells which were, thus, recovering from their deficiency. At longer times, which depended on the amount of phosphate made available, the content per cell decreased because of cell division. In this experiment the amount of phosphorus in the cell varied from 2 to 66×10^{-15} mole/cell, emphasizing again the wide range of variation which can be induced by varying the external conditions. The initial rates of assimilation under these conditions varied from 12 to 24×10^{-17} mole/cell·min. and decreased as the phosphate was removed from solution.

In the second experiment (Table V) phosphorus-deficient cells were allowed to assimilate varying amounts of added phosphorus in the dark so that at the start of the experimental period practically all of the phosphorus was in the cells, their content being from 6.1 to 34×10^{-15} mole/cell. Radio- and carrier phosphate was added in amounts that increased the low concentrations of dissolved phosphate but that were small compared to the phosphorus in the cells. Under these conditions the uptake of phosphate varied from about 1 to 3×10^{-17} mole/cell·min. with no apparent relationship to either the cell phosphorus or the dissolved phosphate. Although the available phosphate concentration in the two experiments varied initially by nearly four orders of magnitude, the rates of assimilation differed by only one.

Phosphorus is not only assimilated rapidly by deficient cells, but intracellular phosphorus is also constantly exchanged with that in the water (Kamen and Spiegelman, 1948; Goldberg *et al.,* 1951; Rice, 1953; and Knauss and Porter, 1954). It was not possible to separate the two mechanisms, uptake and exchange, in the present experiments. The results given in Table V for the assimilation from low phosphate medium were calculated assuming all of the change was uptake with no exchange. A calculation was also made assuming that all can be attributed to exchange, with no net uptake (Russell, 1958), and this gave almost exactly the same rates.

Phaeodactylum stops dividing when the intracellular phosphorus falls to about 2×10^{-15} mole/cell. During its most active growth a population can double in about 18 hours of continuous illumination. The lowest rate of uptake we have measured, 10^{-17} mole/cell·min., would permit a phosphorus-deficient cell to double its phosphorus content in 200 minutes, or slightly over three hours. Since this rate of uptake was from very low phosphate concentrations in the medium, it seems unlikely that the rate of assimilation of phosphorus would ever limit the rate of growth of a phytoplankton population in nature. The total supply could, of course, determine the final size of the population.

The rate of uptake under the different conditions must reflect the physiological state of the cells and must ultimately be dependent upon both the supply of phosphorus in the cells and in the medium, although these relationships are not conclusively demonstrated in our experiments. On the other hand, if availability of intracellular energy and enzyme reserves, needed for the work of active transport through the cell membrane, strongly influences the uptake rate, then darkened cells can have rates nearly those of illuminated ones if they can draw upon previously stored reserves. Odum *et al.* (1958) similarly showed that illumination had little effect upon the P^{32} uptake of benthic algae.

Munk and Riley (1952) showed theoretically that small cells should absorb nutrients more rapidly than larger ones, and Odum *et al.* (1958) demonstrated that filamentous or thin benthic algae absorb P^{32} much faster than the more massive algae. There were, undoubtedly, some differences in cell size and surface-to-volume ratio in *Phaeodactylum,* but these differences probably affected the uptake rates only slightly.

In *Phaeodactylum* the chromatophore shrinks to one-third or less of its original length as the cell becomes phosphorus-deficient. Chlorophyll measurements in other experiments (unpublished) prove that the absolute amount of chlorophyll per cell declines markedly in phosphorus-deficient cells (*cf.* Ketchum *et al.*, 1958). Uptake rates per unit of protoplasm, cell nitrogen, or chlorophyll may prove to be more meaningful than per cell, especially when comparing the rates of algae of widely differing sizes or when studying natural mixed populations. In experiments VI–X, chlorophyll concentration was measured; it was initially 7.1 ± 0.6

$\times 10^{-14}$ g. Chl A/cell, and the uptake rates on this basis range from 1.6×10^{-4} to 4.5×10^{-4} mole P/g. Chl A·min.

Phaeodactylum is able to remove phosphate to levels below the sensitivity of the chemical analytical method. The lowest concentration, determined from P^{32} remaining in solution, was 7.2×10^{-10} *M*. This confirms the often quoted ability of algal cells to concentrate phosphorus greatly. We have not measured the volume of *Phaeodactylum* cells, but Ketchum and Redfield (1949) give a dry weight per cell of 2.32×10^{-11} grams. Assuming 20% dry matter in the cells and a density of 1, the volume of cells in experiments VI–X (Table V) would be 1.9×10^{-2} ml./l. Lewin *et al.* (1958) found very similar weights and volumes in fusiform *Phaeodactylum*. The concentration of phosphorus within the cells varied from 0.08 to 0.3 *M*. These were in equilibrium with external concentrations of 7.2 to 22×10^{-10} *M* at the end of the experiment (Table II), producing concentration factors of about 10^8. Under these conditions most of the intracellular phosphorus probably is firmly bound in the cells with only a very small fraction present as free phosphate ions.

The physiological condition of natural phytoplankton with regard to nutrients is still difficult to assess. The uptake rate even at low concentrations is high enough so that phosphorus should never be limiting in any waters with chemically detectable phosphate concentrations. Our experiments have shown, however, that the chemical method in use measures some materials which are not treated as free phosphate ions by the living cell. Also, *Phaeodactylum* can accumulate thirty times as much phosphorus as the minimum required for cell division and may reduce the concentration of phosphate to undetectable levels in the water while the cells are still relatively phosphorus-rich. Such cells can continue to divide, in the light, with no further phosphorus accumulation. Inability to measure phosphate in sea water, then, can not be taken as evidence that it is limiting population growth or organic production.

SUMMARY

Portions of a phosphorus-deficient culture of *Phaeodactylum tricornutum* Bohlin were dispensed into fresh media containing phosphate concentrations from 8 to 80 μM. The instantaneous initial phosphate uptake rates were 12 to 24×10^{-17} mole/cell·min. The concentrations of phosphorus in the cells extended from a high of 66×10^{-15} mole/cell after 12 hours' exposure to phosphate-rich (32 and 80 μM) media to a low of 2×10^{-15} mole/cell when PO_4 depletion of the medium limited further growth. In another experiment *Phaeodactylum* was prepared with varying intracellular P concentrations in media with very low PO_4 concentrations. Radioactive phosphate was then added, the time course of P^{32} distribution was followed, and the rate of phosphate uptake was calculated. The initial rates ranged from 10^{-17} to 3×10^{-17} mole/cell·min. These were about one order of magnitude less than the uptake rates during the first two hours of the first experiment, even though the PO_4 concentrations were two to four orders of magnitude lower. Radioisotope analysis showed that *Phaeodactylum* decreased the phosphate in the medium to as little as 7.2×10^{-10} *M*, a concentration much below the limit of sensitivity of the chemical analytical method.

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SURVIVAL AND MOVEMENTS OF THE FLATWORM, STYLOCHUS ELLIPTICUS, IN DIFFERENT SALINITIES AND TEMPERATURES

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The flatworm, *Stylochus ellipticus,* an experimentally-proven predator of oysters (Loosanoff, 1956), is one of the most abundant marine polyclads along the Atlantic and Gulf coasts of the United States (Hyman, 1940) and, consequently, may be one of the most important oyster enemies. A review of the literature offered by Hopkins (1949, 1950) showed that there is little published information regarding its physiological behavior. However, Pearse and Wharton (1938) in their report on the physiology of the related species, *Stylochus inimicus,* from Apalachicola Bay, Florida, included some observations on *Eustylochus meridionalis* (*Stylochus ellipticus*) from the same area. They found that *S. ellipticus* could survive a slow decrease in salinity from 32 parts per thousand to as low as 2.9 ppt, but died in salinities below 6 ppt if the decrease was abrupt. They also reported that the worms became dormant at temperatures below 7° C. These circumstances suggested that some environmental control of *S. ellipticus* might exist in certain areas in cold climates, especially where low salinity and low temperature coincide.

Since information on the physiological behavior of *S. ellipticus* in the northern part of its range is lacking, our studies were initiated to observe the behavior of adult worms (10 to 18 millimeters long) of Milford Harbor, Connecticut, at different salinities and temperatures, with emphasis on observations at low temperatures and salinities. While the salinity of Long Island Sound proper usually fluctuates within a narrow range of approximately 26 to 28 ppt, oyster beds located in estuaries, salt water ponds and rivers may at times, especially in the early spring, be exposed to water that is almost fresh. The annual range of water temperature in this latitude is from about − 1° C. to approximately 25° C.

Effects of salinity

Observations were made on survival of the worms in different salinities and the effect of these salinities on movement of these worms. "Righting time," *i.e.,* the time required for a worm to return to normal position after having been turned ventral side up, was used as a quantitative measure. Righting times were determined frequently at each salinity tested; however, more observations were made at the lower salinities, where individual variations in righting time were largest, than at the higher salinities. All observations were made in the laboratory at room temperature (18° C. to 22° C.) in standing water, which was changed twice a week. The low salinities were made by diluting sea water from Milford Harbor (about 27 ppt) with tap water demineralized by a Barnstead BD-2 apparatus.

In the first experiment the effects of an abrupt decrease in salinity on survival and righting time of *S. ellipticus* were investigated. Groups of 10 worms each were transferred directly from Milford Harbor water to enamel pans, arranged in pairs, each containing six liters of water of the following salinities: 25, 22.5, 20, 17.5, 15, 12.5, 10, 7.5, 5 and 2.5 ppt and fresh water. Two groups, each containing 10 worms placed in undiluted water from Milford Harbor, served as controls.

Worms transferred directly to salinities as low as 7.5 ppt survived this abrupt change. Those transferred to salinities of 10 ppt showed no distress at any time,

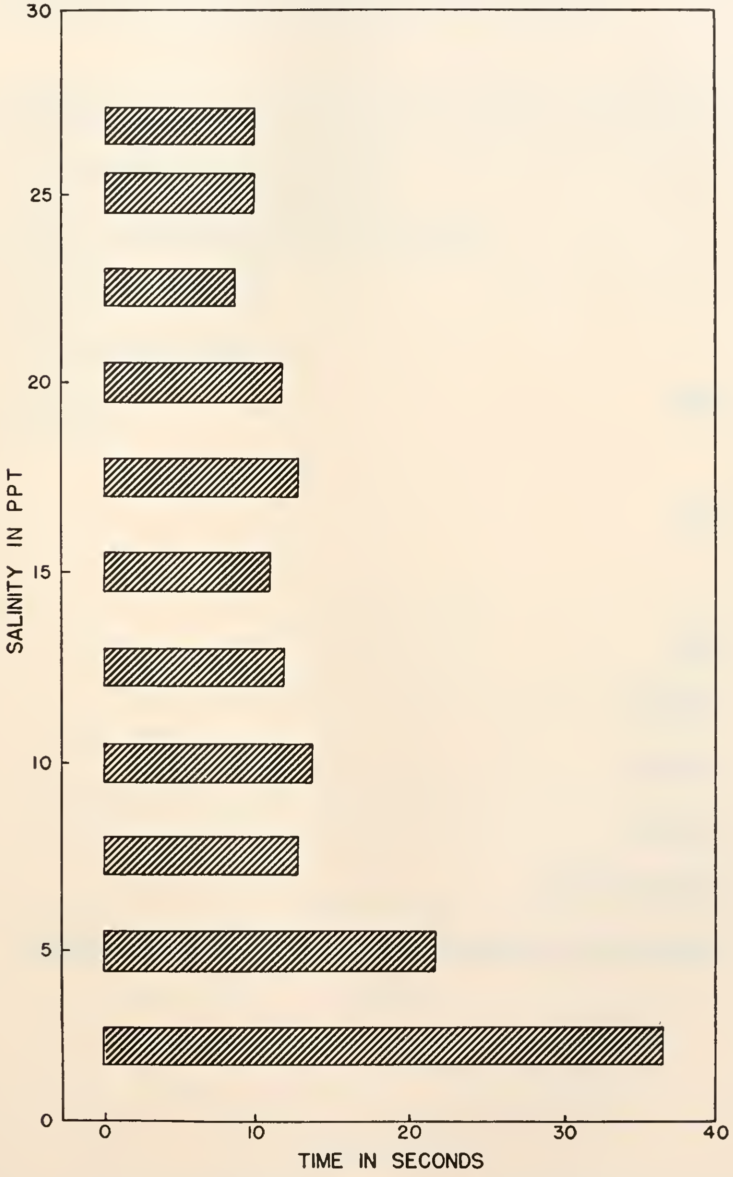

FIGURE 1. Average righting time of *Stylochus ellipticus* from Milford Harbor, Conn. in different salinities at room temperature (18°–22° C.).

while in a salinity of 7.5 ppt some worms, during the first two days, lost color, secreted slightly more mucus than is normal and were sluggish. The worms transferred to 5 ppt were similarly affected, but for a longer time. Four of these died during the first week after transfer, but the survivors eventually ceased to show symptoms of distress and assumed a normal appearance. All of the worms transferred directly to 2.5 ppt and fresh water died within a few hours. However, when some that had become acclimated to a salinity of 5 ppt were transferred to 2.5 ppt, they showed no signs of distress and remained alive and active.

After all symptoms of distress had disappeared in the low salinities, righting times were determined for worms of all groups. The average righting time ranged from 10 to 13 seconds in all salinities, except 5 ppt and 2.5 ppt. In 5 ppt it was 22 seconds and increased to 37 seconds in 2.5 ppt (Fig. 1).

In a second experiment, survival of worms in salinities below 5 ppt was determined after they had been conditioned at intermediate salinities. Three groups of 100 worms each were conditioned for two weeks; one group, at a salinity of 15 ppt;

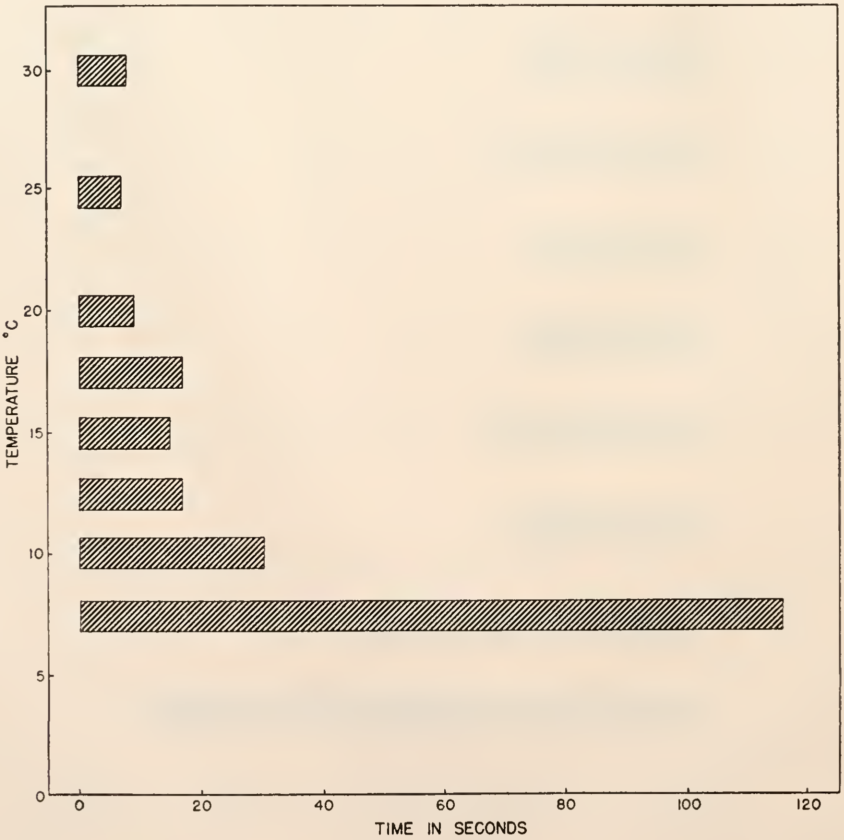

FIGURE 2. Average righting time of *Stylochus ellipticus* from Milford Harbor, Conn., at different temperatures in a salinity of about 27 ppt.

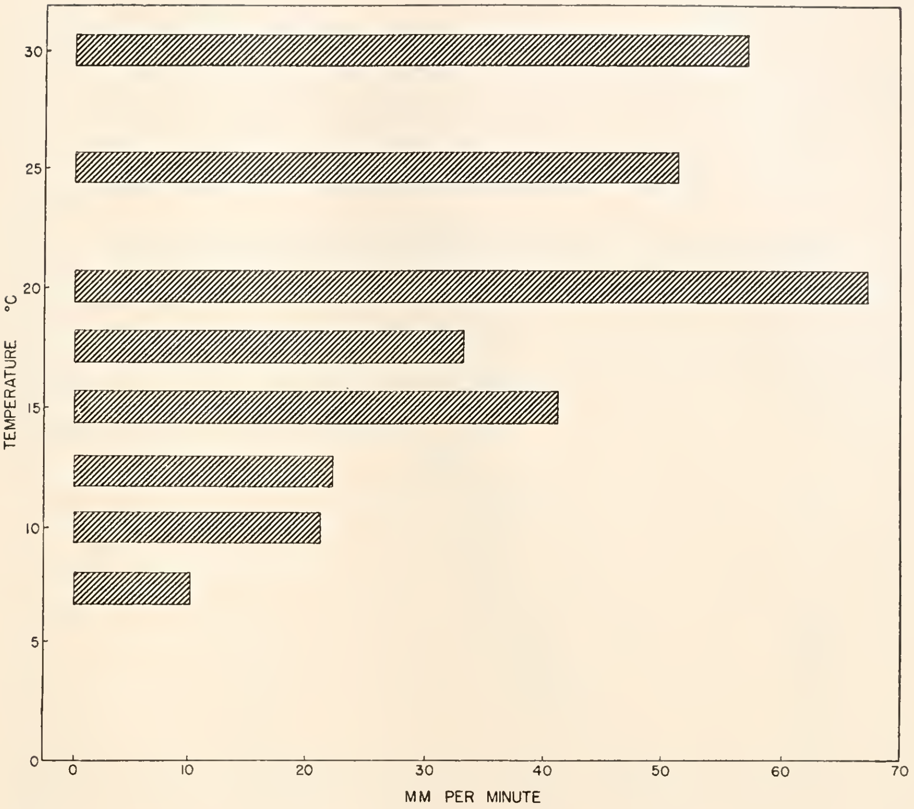

FIGURE. 3. Average locomotion rate of *Stylochus ellipticus* from Milford Harbor, Conn., at different temperatures in a salinity of about 27 ppt.

another group, at 10 ppt; and the third, at 5 ppt. Early in the conditioning period 16 of the worms in 5 ppt died, but the remainder of this group became acclimated. None of the worms died or showed distress in either 15 ppt or 10 ppt. At the end of the two weeks of conditioning, groups of ten worms from each conditioning salinity were placed in duplicate enamel pans, each containing six liters of standing water of the following salinities: 2.5, 1.5 and 0.5 ppt and fresh water. Two groups of ten worms each, placed in undiluted Milford Harbor water, served as controls.

Conditioning at intermediate dilutions had little effect on the ability of these worms to survive in low salinities. By the end of the fourth day all the worms conditioned for two weeks at 15 ppt and then transferred to salinities of 2.5 ppt and lower had died. Worms conditioned at 10 ppt had died by the end of the seventh day after transfer. Those conditioned at 5 ppt, and transferred to 1.5 ppt and 0.5 ppt and fresh water, also were dead by the end of the seventh day. Twelve of the 20 worms conditioned at 5 ppt and transferred to 2.5 ppt died by the end of the seventh day, but the remaining eight were alive and healthy when the experiment ended. Only one of 20 worms in the control pans died during the experiment.

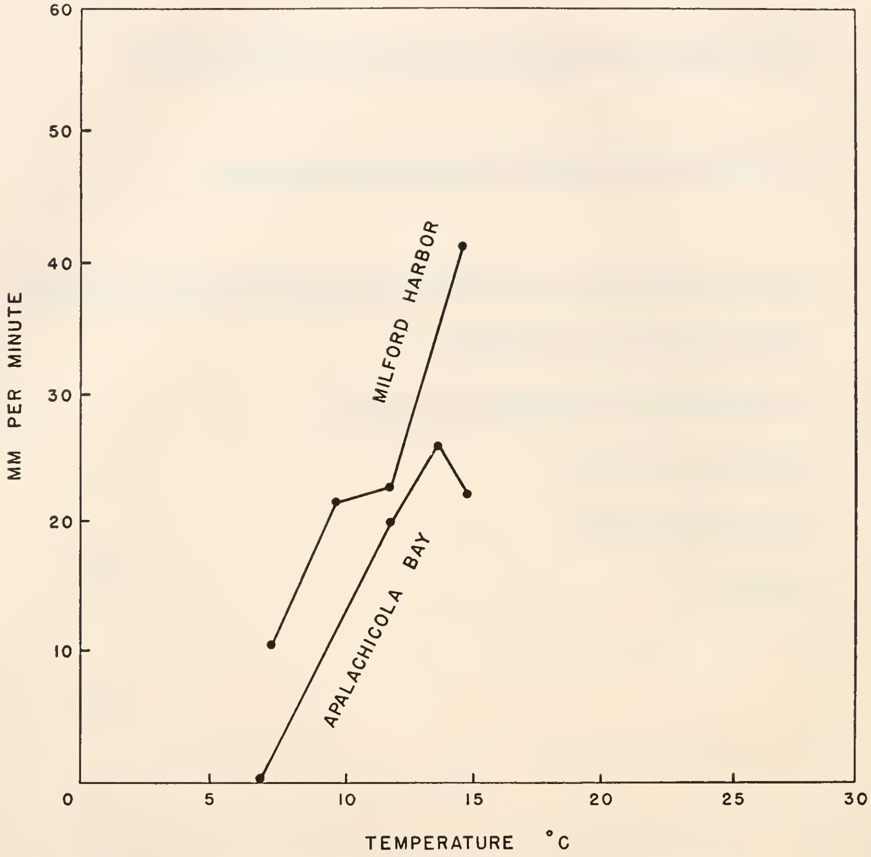

FIGURE 4. Average locomotion rates of *Stylochus ellipticus* from Milford Harbor, Conn., and from Apalachicola Bay, Florida at similar water temperatures.

Apparently, the lowest salinity in which *S. ellipticus* of Milford Harbor can survive, even with prior conditioning at an intermediate salinity, is about 2.5 ppt.

Effects of temperature

The effects of temperature on righting time and "locomotion rate," *i.e.*, the rate of forward movement, of *S. ellipticus* were observed in running sea water at 30°, 25°, 20°, 17.5°, 15°, 12.5°, 10° and 7.5° C. The water temperatures were maintained within ± 1° C. of the desired levels by mixing, in different proportions, heated and unheated Milford Harbor water.

Three groups of ten worms each were allowed to adjust to a selected temperature for three days. Within the next week three separate observations were made on righting time and locomotion rate of all of the worms. At the end of this time the water temperature was changed, the worms were allowed to adjust to the new temperature, and a new series of observations was made. Locomotion rate was determined by noting the length of time needed for each worm to move 40 mm., without stopping, in a straight line.

The average righting time at 20°, 25° and 30° C. was fairly constant at 7 to 9 seconds, but increased to 17 seconds at 17.5° C. and remained at approximately this level at temperatures down to and including 12.5° C. At 10° C. righting time increased to 30 seconds and rose sharply to 116 seconds at 7.5° C. (Fig. 2).

The average locomotion rate varied somewhat erratically at different temperatures, but tended to decrease with decreasing temperature. From a rate of over 50 millimeters per minute at 20° C. and higher it dropped to approximately 10 mm./min. at 7.5° C. (Fig. 3). A comparison of average locomotion rates observed in this experiment with similar data reported by Pearse and Wharton (1938) for *S. ellipticus* from Apalachicola Bay showed that, at the same temperatures locomotion rates of Milford Harbor worms were consistently higher than those of Florida worms (Fig. 4). This difference may indicate the existence of physiological races within this species of flatworm.

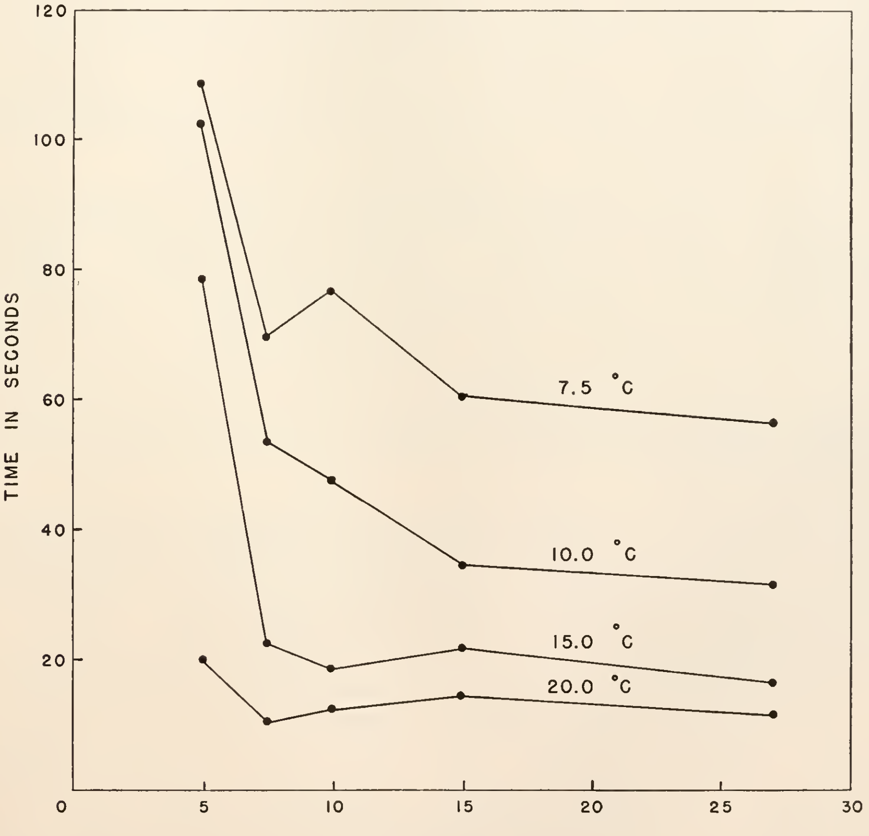

FIGURE 5. Average righting time of *Stylochus ellipticus* from Milford Harbor, Conn. at different salinities and temperatures.

Combined effects of temperature and salinity

The combined effects of temperature and salinity on survival and movement of *S. ellipticus* were also studied. Again, righting time was used as a criterion of movement. Duplicate groups of five worms each were held in polyethylene boxes in three liters of standing water of different salinities. These boxes were kept in a water bath where the desired temperatures were maintained.

The procedure used to study the combined effects of temperature and salinity on the righting times of worms was similar to that used in observing the effect of temperature alone, *i.e.,* worms in all salinities were held at the same temperature until their righting times had been determined, then the water temperature was changed and the observations repeated. Using this procedure righting times were obtained in Milford Harbor water at a salinity of about 27 ppt and also in salinities of 15, 10, 7.5 and 5 ppt at temperatures of 20°, 15°, 10° and 7.5° C. Worms tested at 5 ppt were conditioned to this salinity prior to starting the experiment.

Although worms showed some movement in all combinations of temperature and salinity tested, the effects of low temperature and salinity in slowing down their movements became more apparent as the two factors were progressively lowered, and the depressing effects of each factor on their movement tended to reinforce one another (Fig. 5). For example, at 20° C. worms in all salinities from Milford Harbor water to that of 7.5 ppt had approximately the same average righting time, ranging from 11 to 15 seconds, but worms in 5 ppt required an average of 21 seconds to turn over, reflecting primarily the adverse effect of low salinity.

At 15° C. the average righting times of worms in 7.5 ppt and higher salinities were again about equal, but had increased slightly to approximately 18 seconds, due to the effect of the lower temperature. In 5 ppt, however, righting time increased more sharply than expected. For example, at normal salinity a decrease in temperature to 15° C. had no appreciable effect on righting time, and at room temperature lowering of the salinity to 5 ppt had only increased righting time to 22 seconds, but when the temperature was reduced to 15° C. and the salinity to 5 ppt, simultaneously, righting time increased to 79 seconds. The combined effect was, therefore, greater than could have been predicted from the effects of these factors when studied separately.

At 10° C. worms in all salinities were affected by lowered temperature, while those in salinities of 5, 7.5 and 10 ppt showed the exaggerated effect of a combination of low temperature and salinity. For example, in Milford Harbor water and in 15 ppt the average righting time was 32 and 35 seconds, respectively, while at 10 ppt it was 48 seconds, 54 seconds at 7.5 ppt and 103 seconds at 5 ppt.

At a temperature of 7.5° C. the worms in all salinities had slowed their movements still further, but the same pattern of increase in righting times was noted.

In many areas where oyster cultivation is carried on, pronounced variations occur in either temperature or salinity or both. If the rate of predation of flatworms on oysters is closely related to the worms' ability to move about, our studies indicate that predation by these worms must vary considerably at different seasons of the year, especially in a cold climate. Even though their predatory activities may be curtailed by low temperature or low salinity, their ability to survive these adverse conditions makes them a serious threat wherever oyster culture is practiced.

We wish to express our appreciation to Dr. V. L. Loosanoff for suggesting this problem and for his critical review of the manuscript. We also wish to thank Mr. Harry C. Davis for his helpful suggestions in the preparation of this paper, Mr. Manton Botsford for the illustrations and Miss Rita Riccio for her careful editing.

SUMMARY

1. *Stylochus ellipticus* from Milford Harbor, Connecticut, survived abrupt transfer from a salinity of about 27 ppt to salinities as low as 7.5 ppt at room temperature. Those transferred directly to 5 ppt suffered a mortality of 20% but all worms died when placed directly in 2.5 ppt and fresh water. However, worms that acclimated to 5 ppt survived subsequent transfer to 2.5 ppt.

2. Righting time of *S. ellipticus* at room temperature remained constant at 12 to 15 seconds in salinities ranging from about 27 ppt to 7.5 ppt but increased to 22 seconds in 5 ppt and to 37 seconds in 2.5 ppt.

3. Righting time of *S. ellipticus* in a salinity of about 27 ppt was approximately 8 seconds at temperatures of 20°, 25° and 30° C., 16 seconds at 12.5°, 15° and 17.5° C., 30 seconds at 10° C. and 116 seconds at 7.5° C.

4. Locomotion rate of *S. ellipticus* varied erratically with temperature but generally decreased with temperature decreases below 20° C. It exceeded 50 millimeters per minute at temperatures of 20° C. and higher but decreased to 10 mm./min. at 7.5° C.

5. At the same temperatures *S. ellipticus* from Milford Harbor moved faster than reported for the same species from Apalachicola Bay, Florida. This observation suggests that there may be physiological races within this species of flatworm.

6. When temperature and salinity were lowered simultaneously righting time of *S. ellipticus* was frequently longer than the combined righting times obtained when the two factors were observed separately.

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BODY TEMPERATURES IN SOME AUSTRALIAN MAMMALS. III. CETACEA[1] (MEGAPTERA)

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Whales have always attracted popular interest both as the largest animals and as a valuable object in commerce, but their size and wide range make approach difficult and have discouraged the accumulation of reliable information about them. Although their unique size lends interest to any aspect of their life, the topic of body temperature to be considered here bears on a point of particular interest in view both of the general relations between energy production, mass, and heat dissipation in mammals, and the special problems of aquatic mammals (Scholander and Schevill, 1955).

Because of "technical" difficulties, body temperatures have only conveniently been measured on dead whales, although advances in telemetry may offer another approach in the future. Ordinarily one would consider a measurement obtained an hour after death to be quite questionable and one obtained ten hours after death to be of no value at all. However, the enormous bulk of the whale greatly reduces heat dissipation. The covering layer of blubber constitutes an effective insulation and this, together with the high heat capacity of the system and the low relative surface area, greatly reduces heat loss. It has been estimated that a 24 M. (122-ton) whale has a surface of 275 M.^{2} or only 23 cm.^{2}/kg. (Laurie, 1933). This is a tenth of the value in man and less than a hundredth of that in a mouse. Of course, in the living animal most of this reduction in surface is compensated for by the (presumed) lower metabolic rate in the whale, so that the metabolic output per unit of body surface may differ by no more than a factor of three in the largest and smallest mammals.[2]

In practice, it has been long known that whales do cool slowly after death. For example, Guldberg (1885) reported a temperature of 34° in a fin whale three days after death. Cockrill (1951) described a whale fillet of about three tons which was towed for 21 hours in antarctic waters with a resultant fall of only 1° F. in deep temperature. Ash (personal communication) has "observed

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[1] This study was carried out with assistance from the Guggenheim Foundation and the U. S. Educational Foundation in Australia, and would not have been possible without the wholehearted assistance of Dr. Victor Macfarlane of the School of Physiology at Brisbane. I am indebted to Dr. Masaharu Nishiwaki of The Whales Research Institute (Tokyo), to Dr. J. G. Sharp of The Low Temperature Research Station (Cambridge) and to Dr. C. E. Ash of United Whalers Ltd. (London) for supplying very interesting unpublished records of whale temperatures; and to Dr. Clinton Woolsey for similar data on a porpoise. I also appreciate the kindness of Dr. William E. Schevill in checking the manuscript and nomenclature.

[2] This represents the difference between the two weight functions for surface ($A = kW^{.667}$) and metabolism ($M = kW^{.734}$) so that $M/A = kW^{0.067}$. Taken over the shrew-to-whale weight-span ($3 \rightarrow 10^8$ g.) this amounts to a factor of 3×. Over the mouse-to-elephant span ($20 \rightarrow 4 \times 10^6$ g.) the factor is 2.2.

a fillet of meat (longissimus dorsi) maintain a steady temperature of 35° C. in the interior for 15 hours *post mortem.*" Robinson *et al.* (1953) summarized data to show (p. 6) that "up to 24 hours after death, whale muscle does not lose heat to a significant extent." Indeed, Irving and Krogh (1954) have reported that even in the very much smaller (though well insulated) reindeer and caribou, and under the extreme ambient conditions of −45° C. in strong wind, there was no demonstrable fall in deep body temperature in the hour following death.

Materials and Methods

Observations were made on 20 recently killed humpback whales (*Megaptera novaeangliae*) at coastal whaling stations on Moreton Island in southern Queensland just off Brisbane and in Byron Bay in northern New South Wales, the easternmost point in Australia. We are indebted to the respective concerns, Whale Products, Ltd. and the Byron Whaling Co., Ltd., for their cooperation in making these studies possible. Measurements on four whales (No. 512, X, Y, and Z) were kindly made by Dr. K. C. Robbins of the CSIRO. At both these locations whales are killed close offshore from small vessels similar to those used in the larger antarctic whaling operations. But instead of being picked up by a large "factory" ship they are towed to fixed shore installations where the carcasses are cut up and rendered. The period between death and disposal varies from one to sixteen hours, depending on the towing distance, the weather conditions, and the work load. These whales ranged from 35 to 45 feet in length and were considered to carry roughly a ton of weight for each foot in length.

Temperatures were measured with copper-constantan thermocouples using a Cambridge thermocouple-potentiometer unit. This provided connections for manual switching between six thermocouples. One of these was used as a reference junction for calibration of the instrument against a mercury thermometer in a small Dewar flask. The other five were fixed at twelve-inch intervals along a one-inch stainless steel tube. The plastic-coated thermocouple wires were further protected by an outer small plastic tube, which was led down the inside of the probe and brought out at the appropriate point through a small hole. The junction was thus exposed on the outer surface of the probe (although still protected by the plastic cover), and the far end was run through another hole and secured inside by knotting. A demountable harpoon head with tempered cutting edges was fixed to the shaft to allow penetration through the tissues. The first thermocouple lay two inches above the head, and the fifth, four feet higher; but all thermocouple wires were of the same length. In preliminary tests it was not possible to check the thermocouples against one another to better than 1° C. But on two occasions during measurements on whales the heart was penetrated so that the hollow thermocouple probe carried a stream of blood out of the body. Under these conditions, values for the several couples all check each other within 0.1° C.

In one series of measurements, made from the deck of the whale chaser immediately after capture, approach was limited to the more posterior regions and insertions could be made only with great difficulty. Because of unfavorable weather conditions, the carcass rose three to six feet with each swell, and on several occasions the one-inch steel probe was bent to a right angle against the side of the vessel. Most of the measurements, however, were carried out after the whales had been towed ashore and under these circumstances the probe could be readily positioned in any part of the body. Insertions were normally made from the ventral surface near the midline, with the last thermocouple set just below the surface, or when further penetration was stopped by bone. The various insertion positions are diagramed in Figure 1.

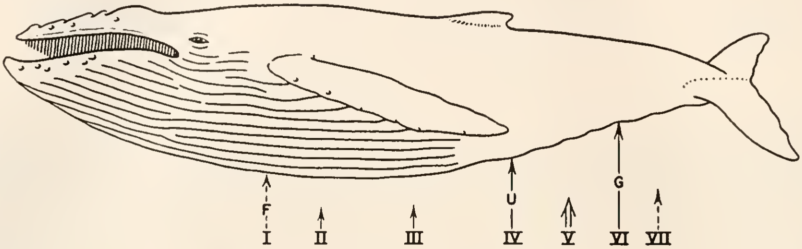

FIGURE 1. Humpback whale profile showing axial positions for thermometer insertions. F, flipper; U, umbilicus; G, genital opening.

RESULTS

In each case, values from the four or five thermocouples provided a temperature profile through the whale. The anticipated sequence, that most commonly

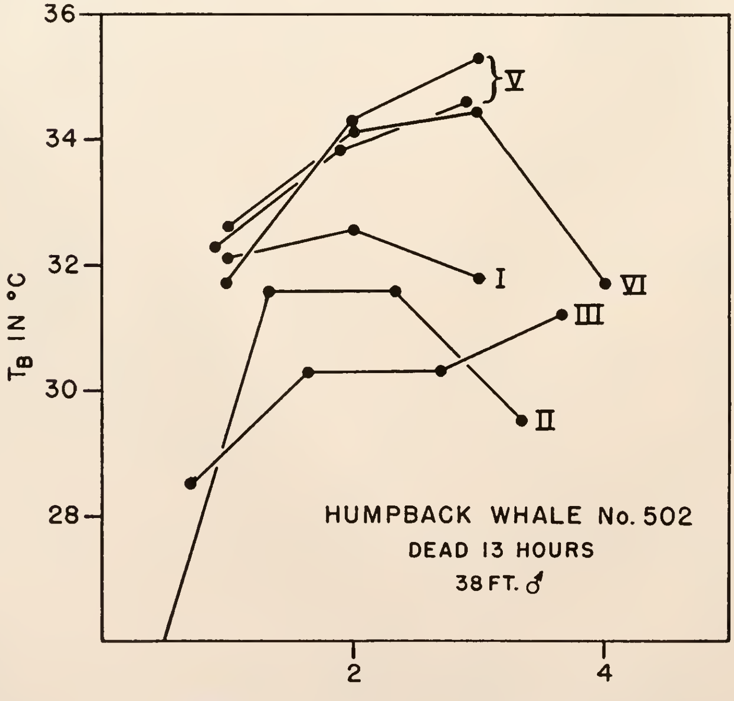

FIGURE 2. Temperature-depth profiles at various axial positions in whale No. 502, showing normal gradients.

observed, found the innermost couple (#1) or perhaps the next (#2) at the highest temperature, with the other values decreasing uniformly as the surface was approached. A representative set of such data is shown in Figure 2.

However, sometimes quite abrupt changes were observed between adjacent regions; and even reverse gradients were seen (Fig. 3). The reason for these marked changes in temperature was not immediately evident, although a somewhat similar lability was described by Nishiwaki (personal communication) in successive measurements on whales taken during Japanese operations in the Antarctic. Substantial changes in temperature either over short distances or short time periods seem incompatible with either a thermoregulated or an inert body of this bulk. The explanation appears to lie in changes which occur during capture. At this time, the whale is first secured by a harpoon with an attached line; and a subsequent shot carrying an explosive charge kills the animal. Sometimes several such shots may be required. These wounds can admit sea water into the animal; and during their death throes quantities of sea water may be ingested, taken into the rectum or vagina, or aspirated into the lungs.[3] Accordingly, the temperature profile through positions III and V in Figure 3 must reflect the presence of a mass of cold water, in this case at a depth of 1.5 to 2.5 feet from the surface.

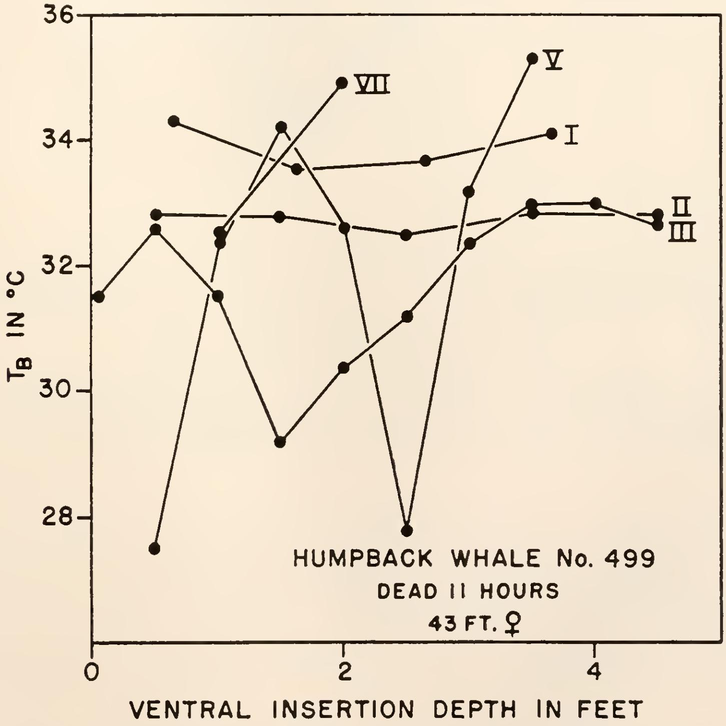

FIGURE 3. Temperature-depth profiles at various axial positions in whale No. 499, showing inverted gradients.

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[3] Such circumstances were described by men of the flensing crew.

Body temperatures also varied markedly with the location of the insertion along the body axis (Fig. 4). Highest values were found to the posterior, and a point of insertion midway between the umbilicus and the genital opening consistently gave the highest readings. At this point, the inner thermocouples would lie in or near the large muscle masses along the vertebrae. Mean maximum values at the various insertion positions are shown in Figure 4. Values at the level of the heart (position II) were somewhat higher than the next point to the rear, but averaged 2° lower than the maximum for the animal (position V).

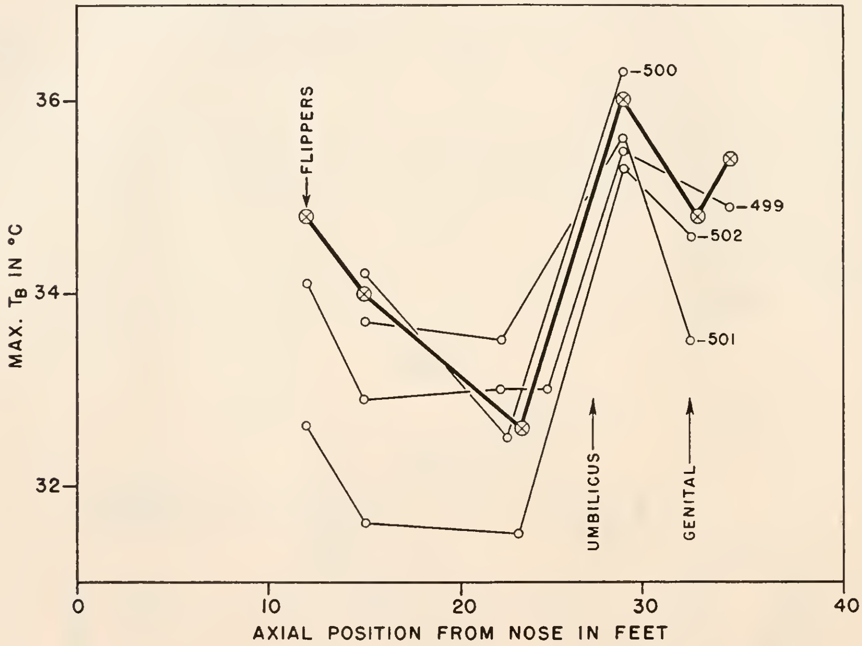

FIGURE 4. Maximum body temperatures at various axial positions in four individuals. Heavy curve through crossed circles represents an average for all values calculated from means for pairs of values from two axial positions (all axial positions were not measured on all whales).

Complete data for these maximum temperatures along the axis are presented in Table I. Data for the entire series of whales, including the maximum temperature observed anywhere in the body, are summarized in Table II. No relation is seen between body temperature and either sex or length. Nor is there a correlation with the time after death, mean values after 2, 8, and 14 hours differing by only 0.2° C.

Average values for all points in each transect are presented in Table III. It is of interest that, even though position V always produced the highest single temperature, the mean value along this transect was lower than that at either the genital or the umbilical position on each side.

TABLE I

Maximum temperatures at various axial positions

Whale	I & II	III	IV	V	VI & VII
492	35.2	35.6	35.6		
493	34.8	34.4	34.1		35.9
494		35.2			
498		32.1		36.3	
499	34.3	33.0		35.5	34.9
500	34.2	32.5		36.3	
501	33.7	33.5		35.8	33.5
502	32.6	31.5		35.3	34.6
503	34.9		35.1		35.3
512				36.1	
X				36.2	
Y				36.8	
Z				37.1	
B	34.5	36.1		35.3	35.4
C				35.0	
D	34.0	33.7			34.5
G			37.3		37.7
H					36.8
I	35.7	35.8		36.3	34.3
J	34.9			34.9	34.2
Mean values	34.4	33.9	35.5	35.8	35.2
	(11)	(11)	(4)	(14)	(11)
Ave. depth (ft.)	2.7	3.8	3.7	3.3	2.8

DISCUSSION

This study has shown that there may be considerable variation in temperature in different parts of the whale's body as measured after capture by commercial procedures. Some of this variation appears to be an artifact resulting from the intake of cold sea water into the body. However, one may minimize this error, as was done, by multiple testing to identify and avoid any "cold spots." But, with this precaution taken, there still remain substantial differences in the maximum temperatures along the body axis. Such axial temperature gradients are not uncommon among other mammals and may be quite prominent in some special situations such as arousal from hibernation; but the region of highest temperature is always near the heart and liver, with values declining to the rear. In the whale we see the reverse situation, with the higher values at the rear and lower values to the front. Values in the heart itself are not the lowest, but average 1.5° C. below the maximum. This may reflect a different balance in this large animal between the heat production in the muscle mass and that in the visceral organs. In considering this question, it would be very useful to have values for brain temperatures in whales and to have axial distributions in smaller cetaceans, but neither are available. However, measurements on the small killer whale (*Orcinus*) by Portier (1908) and on the seal (*Phoca*) by Scholander *et al.* (1942) gave higher brain temperatures than visceral temperatures. Another possibility to account for this axial gradient is extra heat production by the

TABLE II

Maximum body temperatures in humpback whales

Whale	Sex	Length ft.	Hours dead	T_B °C.
B	♂	37	5	36.1
C			0.5	35.0
D			0.5	34.5
G			1	37.7
H			0.8	36.8
I			5.5	36.3
J			2.3	34.9
			2.2	35.9(7)**
492	♂	40	8	35.6*
493	♂	35	7	35.9*
494	♀	33	7	35.2*
503	♂	35	7	35.3*
X	♀	34	8	36.2
Y	♀	33	8	36.8
Z	♀	36	8	37.1
			7.6	36.1(7)***
512	♀	38	17	36.1
498	♂	41	11	36.3
499	♀	43	11	35.5
500	♀	42	18	36.3
501	♂	39	14	35.8
502	♂	38	13	35.3
			14	35.9(6)***
				36.0(20)

* "U-G" position not measured; may average 0.4° low.
** Whales from Byron Bay.
*** Whales from Moreton Island.

TABLE III

*Average body temperatures at various axial positions**

Whale	I or II Flippers**	III F.-U.	IV Umbilicus	V U.-G.	VI Genital
492	(35.2)	35.1	35.4		
493	34.4	33.6	33.5		35.3
494		34.8			
498		30.8		35.7	
499	32.7	31.5		32.5	34.9
500	32.9				
501	(33.7)	32.8		32.4	32.1
502	(32.2)	31.1		33.8	33.4
503	34.1		34.7		34.3
	33.3	33.0	34.5	33.8	34.2

* 250 temperatures on 21 whales.
** Parenthesized values at flippers; others 2–3 feet behind at heart.

intestinal bacteria. In the largest land mammal, the elephant, the rectal temperature is suggested to be higher than the rest of the body because of this effect (Benedict, 1936). However, as carnivores, the whales should be less subject to this influence; and Robinson *et al.* (1953) report a very low bacterial content in the feces, perhaps 10^{-3}–10^{-6} times that in most fecal material. But, whatever the bacterial calorification, its effect would be enhanced by the large volume available and the whale's lower metabolic rate.

The data of Nishiwaki referred to above show some striking declines in temperature over relatively short periods. Indeed, the average decline for a series of whales is almost 3°/hr. (Fig. 5). It seems impossible to account for this except as a local cooling effect of water taken into the body at the point of measurement.

Equally striking are three curves where the temperature increased sharply instead of decreasing. One wonders if these may represent the phenomenon of

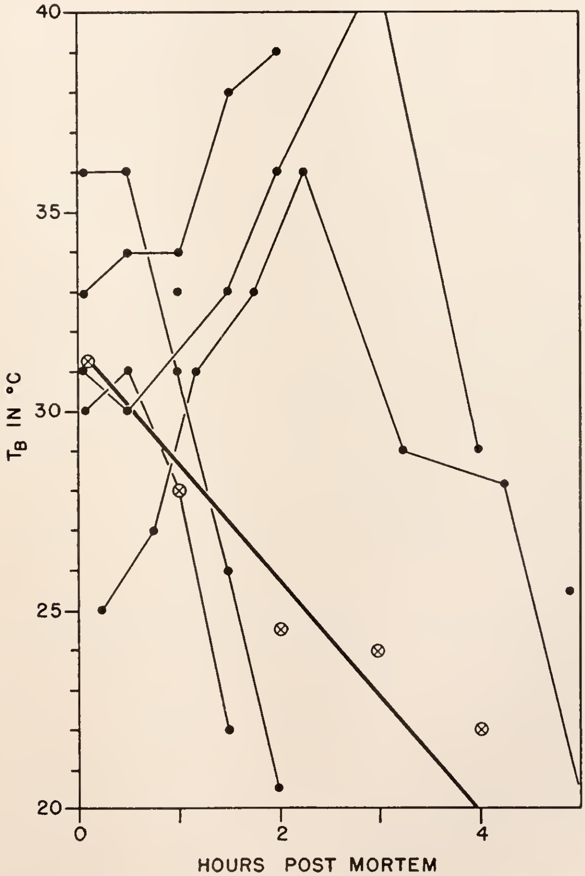

FIGURE 5. Temperature changes in dead whales from data of M. Nishiwaki (personal communication). Heavy curve and crossed symbols represent average of all data. Other curves illustrate rapid transitions of temperature.

"burning," a sharp increase in the dead whale's temperature described by whalers, which may be attributable to an explosive bacterial proliferation through the tissues. Under certain conditions, anaerobic bacteria may be widely distributed throughout the animal, apparently carried from the gut through the blood stream (Robinson *et al.*, 1953). I know of no actual temperature measurements which describe this phenomenon, although Kanwisher and Leivestad (1957) have reported an increase of 0.3°/hr. over an 8-hour period in one whale. The observed increases of 4 to 5°/hr. (Fig. 5) represent about 1 cc. O_2/g. hr. or more than ten times the (predicted) basal heat production of the whale. The observed maxima near 40° (Fig. 5) may seem rather low to justify the impression of "burning," but it should be kept in mind that such comments are made by men acclimated to polar conditions. A temperature field of this size and intensity set in an icy sea could well feel uncomfortably hot to these observers.

TABLE IV

*Whale temperatures by F. H. Addison**

Hours dead**	T_B***	Range†	Values††
1–4	34.5	32–38	54
5–6	34.5	33–38	51
7–8	34.6	32–37	87
9–10	34.7	33–37	90
11–12	34.8	33–37	81
13–14	34.8	33–37	46
15–17	35.1	33–37	81
1–17	34.7	32–38	490

* Unpublished Ministry of Food Report, "Antarctic Whaling Expedition, 1949–50"; personal communication from Dr. J. G. Sharp.

** Observations made on deck of factory ship.

*** "The highest meat temperature is invariably obtained of the longissimus dorsi, the best site being the middle part of the muscles adjacent to the lumbar vertebrae."

† The wide range reflects the large samples. These data showed a normal distribution with a standard deviation of 0.9° C. (See Fig. 6.)

†† The bulk of these measurements (92%) were on Fin Whales, but included 27 values for Blue Whales and 14 for Humpback Whales.

The observations of Addison represent a beautiful series of data to show how constant the body temperature can be in whales (Table IV). The number of these measurements (490) is more than three times that of all the other values identified in the literature. In these measurements on muscles from animals which reached the factory ship at times ranging from one to seventeen hours after death, no fall in temperature at all was seen and actually a small increase (0.033° C./hr.) was observed. This sequence also involved the transition through *rigor* from "dry" meat to "wet" meat, and the increase of 0.6° C. in temperature is attributable to the breakdown of the phosphate energy reserve in the muscle. When values for "wet" and "dry" meat were plotted separately, neither showed any change in temperature with time.

The range of observed values in this series (89–101° F. or 31.7–38.3° C.) seems large but only reflects the large sample size. A frequency polygon of these

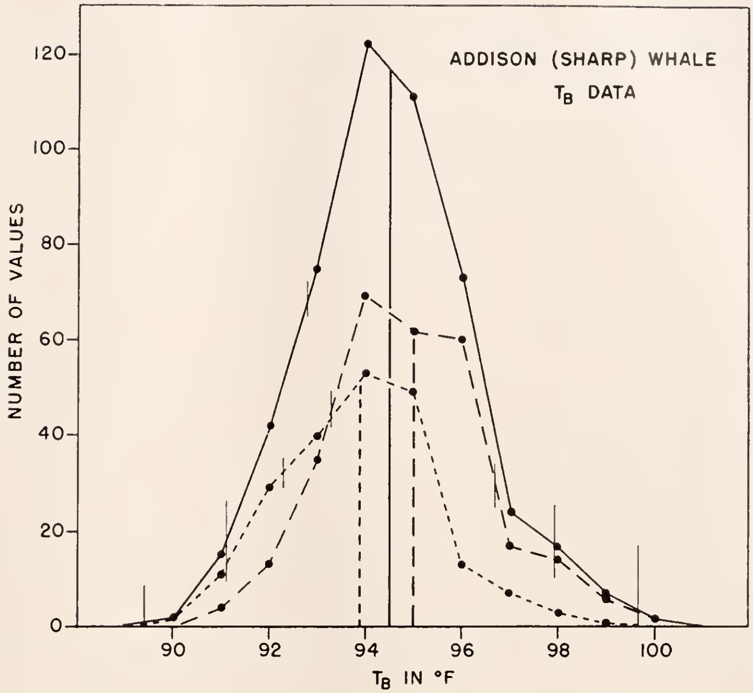

FIGURE 6. Frequency distribution polygon for *longissimus dorsi* temperatures from data of F. A. Addison (personal communication from J. G. Sharp). Heavy curve, all values; dotted curve, "dry" meat (before *rigor*); dashed curve, "wet" meat (after *rigor*): ±1 S. D. = 68%; ±2 S. D. = 94%; ±3 S. D. = 99.6%. The terms "dry" and "wet" are practical expressions describing meat which is respectively firm and sticky (before *rigor*) or wet and sloppy (after *rigor*) to the touch. A third term, "rubbery," relates to muscle actually in *rigor mortis* (Sharp and Marsh, 1953).

data is given in Figure 6 for the total data as well as for the "wet" and "dry" samples. This transformation has an evident, but lesser, influence on the spread of the data, the principal variability being, apparently, differences in initial temperatures in the whales. The standard deviation of the values, 0.9°, represents considerable variation but not more than is seen in many other mammals.

The data of Zenkovic (1938) are of particular interest because he measured five different species and further was able to make observations on five individuals which were still alive, although seriously wounded. Because this paper is difficult of access, the results are summarized in Table V. Ordinarily, values from living animals would be given precedence over values from dead animals. But we have just considered this problem at length to conclude that in the whale this is not so. However, Zenkovic's five values on still living animals are distinctly higher than his other values, including one taken less than an hour after death. Our best judgment is that these "live" values are not representative, but are the result of the continuing exertions of the mortally wounded whale, which is, of course, only approached when it has been completely exhausted and is almost dead. This is not to say that these temperature levels are necessarily outside the normal distribu-

TABLE V

*Whale temperatures from Zenkovic (1938)**

Hours after death	Sperm	Grey	Fin	Humpback	Blue	Mean	Hours after death
0	38.2 (2)		38.4 (2)	38.1(1)		38.3 (5)	0
½–1		36.5 (3)				36.7(12)	½–1½
1–1½		36.4 (6)	37.1 (3)				
1½–2	36.7 (3)			36.3(4)		36.5(12)	1½–4
2–3		36.6 (3)					
3–4			36.2 (2)				
5–7½	35.5 (6)					35.9 (9)	5–9
7½–9		36.3 (3)					
9–13			35.5 (3)				
12–16					35.7(3)	35.6 (6)	9–16
$0 < T_B \lesseqgtr 4$	36.7 (3)	36.6(12)	36.8 (5)	36.3(4)		36.6(24)	
$0 < T_B$	35.9 (9)	36.5(15)	36.3 (8)	36.3(4)	35.7(3)	36.2(39)	
$T_B \lesseqgtr 4$	37.3 (5)	36.6(12)	37.3 (7)	36.7(5)		36.9(29)	
mean T_B	36.3(11)	36.5(15)	36.7(10)	36.7(5)	35.7(3)	36.1(44)	

* "For live whales, insertion was either in the animals side nearer the belly, almost at the beginning of the whales side folds; or sometimes in the belly depending on the position in which the whale was made fast to the boat."

"Thermometer No. 2 was completely immersed to a depth of 30–40 cm in the body and extracted with a line of veins. The thermometers were kept in the animals body for 10 minutes".

TABLE VI

*Whale temperatures by M. Nishiwaki**

Species	Sex	Length ft.	Chase hr.	Hours post-mortem	T_B** (Range)	#
Blue	♂	75–79		<1	33(30–36)	6
	♀	70–87		<1	31(27–35)	7
Fin	♂	62–71		<1	29(25–31)	6
	♀	67–74		<1	32(29–36)	5
				<0.1	31.0(25–36)	21
			<0.2	<0.3	29(25–34)	7
			0.25	<0.3	33(30–35)	3
			0.5	<0.3	32(29–35)	4
			1–2	<0.3	32(30–35)	3
			5	<0.3	36	1

* Taken during the Japanese Antarctic Whaling Expedition, 1948–49 (personal communication).

** Values represent measurements in which a thermometer probe was inserted 60–70 cm. (2½ ft.) into the "trunk, the portion immediately below the base of the flipper." "It is, therefore, probable that the bulb rested in the abdominal cavity in some of the measurements." In five individuals "trunk" values averaged 4° higher than "tail" values in which "the bulb of the thermometer was forced 40–50 cm. (1½ ft.) into the portion of the lateral side of the whole body below the dorsal fin."

tion for whales, since Addison's range of values went as high as 38°. We could, then, take this as an example of the effect of vigorous activity on body temperature in the whale, but would not accept these values as representative of the average temperature in this animal.

Nishiwaki's data (Table VI) may also be looked at in this regard. Comparison of animals chased for times ranging from ten minutes up to five hours suggests some increase, but the variability is too great for conviction. And, of course, an animal could well reflect considerable prior activity, even though it were chased for only a short time. In both these studies average values for the several species showed no significant differences, nor were differences due to sex or size apparent.

Whale temperatures from all available sources are summarized in Table VII and average 35.4° C. for twelve authors. The greater number of these values were specifically taken in the dorsal musculature and average 34.6° C. for 547 individuals (34.5° C. for eight authors). This is a distinctly lower mean than that of the residual group which includes the present study, 36.2° C. for 57 individuals (36.9 for four authors). This suggests that, although consistent high values are found in the back muscle, it is not the warmest spot in the whale body. In the porpoise (*Delphinus*), Richard and Neuville (1897) found the viscera to be 0.3° C. warmer than the dorsal muscle mass.

This overall mean value of 35.4° C., or even the latter mean of 36.2° C. for

TABLE VII

Average body temperatures in whales, after various observers

Observer	Species	Hours dead	T_B °C.	Values	Location
Scoresby, 1820	Bowhead		38.8	(1)	Blood
Guldberg, 1900	(Sperm)*		(40.0)*		
Guldberg, 1885	Blue	2	35.4	(1)	"Back flesh"
Laurie, 1933	Blue, fin		35.1	(30)	*Long. dorsi*
Zenkovic, 1938	Humpback, fin, sperm, blue gray	1–16	36.2	(35)	
	Humpback, fin, sperm	alive	(38.3)	(5)	
Aaser, 1944		1–24	33.1	(18)	Inside muscle
Parry, 1949	Blue	1	35.5	(3)	Epaxial muscle
Cockrill, 1951			(31–34.5)		9″ in fillet
Robinson *et al.*, 1953	Blue, fin	0–24	33.4	(26)	Inside muscle
Kanwisher and Leivestad, 1957	Fin		36.6	(1)	Musc. and body cavity
Addison**	Fin	1–16	34.7	(490)	*Long. dorsi*
Ash**		−15	35	(1)	*Long. dorsi*
Nishiwaki**	Blue, fin	1	(25–36)	(24)	
Sharp**		0.4	34.4	(1)	*Long. dorsi*
This study	Humpback	0.5–18	36.0	(20)	See text

* Guldberg (1900) cited Beal (1839) for this value and species, but Beal only refers to this value as an upper limit for cetaceans. Desmoulins (1822), whom Beal cited for his statement, apparently arrived at the value of 40° C. by arbitrarily adding on 1–4° C. for presumed cooling to the values of Scoresby (1820) for a narwhale and a baleen whale (Tables VII and VIII).

** Personal communication.

whales, is well below the averages of 37.8° C. for 56 temperate mammal species (Morrison and Ryser, 1952) and of 38.3 for 21 Alaskan mammal species (Irving and Krogh, 1954). It is of interest to see if this is also true of smaller cetaceans, *i.e.*, whether the low body temperature relates to the large size or to the order of Cetacea. Table VIII summarizes the temperatures available for smaller cetaceans. Values from nine authors range from 35.6 to 37.8° C. and average 36.5° C. (36.7 for 13 individuals). It might be suggested that abnormally high values

TABLE VIII

The body temperature of some smaller cetaceans‡

Reference	Species	T_B in °C.*	Site	Notes
Richer (1672)	marsoüin	"Scarcely less warm than land animals"**	Abdomen	
Boerhaave (1741)***	fishes with lungs	"As other mammals"		
Broussonet (1785)	marsoüin	35.6†	Neck wound	Bleeding heavily
Scoresby (1820)	*Monodon monoceros*	36.1	"Blood"	15′; dead 90 min.
Davy (1826)	porpoise	37.8	Liver	Live on deck, at lat. 8°
Richard and Neuville (1897)	*Delphinus delphis*	35.6 (35.3)	Rectum, abdomen Dorsal muscle mass	Harpooned
Grieg (1907)	*Orcinus gladiator*	37.1*	Muscle	Harpooned, dragged on shore
Portier (1908)	*Orcinus gladiator*	36.6 (36.9)	Rectum, liver, vagina Brain	
Jolyet (1893)††	*Tursiops truncatus*	37.0		
Wislocki (1933)	*Tursiops truncatus*	36.0		Stranded (?)
Woolsey†††	*Tursiops truncatus*	37.0	Rectum	Restrained out of water for 2 hrs.

‡ Early interest in the temperature of aquatic mammals is notable. Martine (1740), who has been cited as the first reliable authority in medical thermometry, devoted more space to this group than to all the other homeotherms except man.

* All values represent single individuals except Grieg (=5).

** Apparently no thermometer used.

*** Probably not an original observation.

† Calculated from original value of 28.5 taken as °R.

†† Not clear if this is an original measurement.

††† Personal communication.

will be obtained from cetaceans restrained out of water. If the three such references are eliminated, the average for the remainder is 36.2° C. (six authors and individuals).

We might look for a more general correlation of low body temperature with an aquatic mode of life. Values in Table IX for two carnivores average 37.2 (12 individuals) and for seven pinnipeds average 37.3 (70 individuals). These means are closer to the general averages for mammals but are, however, distinctly below a mean value of about 38.5° C. for their terrestrial relatives in the Carnivora which have higher-than-average body temperatures (Morrison and Ryser, 1952; Irving and Krogh, 1954). One further specific comparison of interest relates the marine polar bear to its terrestrial relatives. Twelve values for black and brown bears average 37.9° C. or 0.5° C. above the level of the polar bear (Irving and Krogh, 1954; Hock, 1957). Although the difference is small, it appears statistically significant (t = 3.0). In sum, therefore, all of these group means do support the association of aquatic life with a reduction in body temperature.

TABLE IX

Body temperatures in marine Carnivora and Pinnipedia

Species	TB in °C.	Site	Author
"Sea Calf"	(38.9)	abdomen	Martine (1740)
Phoca vitulina	37.8 (24:3)* (38.4) (7:2)	liver, abd. brain	Scholander *et al.* (1942)
Erignathus barbatus	37.2 (5:5)††	rectal	Irving and Krogh (1954)
Halichoerus grypus	36.5 (6:1)		Scholander (1940)
Mirounga angustirostris	36.0 (13:13)***	rectal	Bartholomew (1954)
Mirounga leonina	37.8		Aretas (1951)
Callorhinus ursinus	37.4 (32:32)	heart	Hanna (1924)
Callorhinus ursinus	37.7 (13:13)**		Bartholomew and Wilke (1956)
Eumetopias jubata	38.5 (2:2)†	rectal	Irving and Krogh (1954)
Enhydra lutris	38.5 (1:1)	rectal	Irving and Krogh (1954)
Enhydra lutris	36.8 (6:6)†		Stullken and Kirkpatrick (1955)
Thalarctos maritimus	37.5 (3:3)†		Anon. (1827)
Thalarctos maritimus	37.3 (2:2)†	viscera	Irving and Krogh (1954)

* Lost 2.5° during dive; parenthesized numbers show measurements and individuals.
** Gained up to 4° during activity on land.
*** Lost 2.2° at night (5:5).
† Shot.

SUMMARY

1. A series of some 250 body temperature measurements were made on 20 humpback whales (*Megaptera novaeangliae*) from the east coast of Australia. The distribution in the animal was plotted by means of a series of ventro-dorsal temperature profiles. Inverted temperature profiles were sometimes found, indicating the presence of internal masses of cold water and offering an explanation for the aberrant temperature values sometimes reported for whales.

2. Maximum values were found posteriorly near the umbilicus and the genital

opening, and at a depth of 3.3 feet. The average was 36.0°; and there was no correlation with sex, size (33–40 ft.), or time after death (0.5–18 hrs.). This body temperature is close to the mean of literature values for whales (35.8°) and, as well, for smaller cetaceans (36.4°), but is appreciably below that for the Pinnipedia (37.3°).

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INTERTIDAL CLUSTERING OF AN AUSTRALIAN GASTROPOD[1, 2]

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The migrations and adaptive behavior of intertidal gastropods have received increasing attention in recent years (Abe, 1955; Jenner, 1958; Anderson, 1961; Fraenkel, 1961; Kornicker, 1961; Sindermann, 1961), but the life histories of cerithiid mollusks are little known (Anderson, 1960), and the small gastropods of northern Australia have received little attention generally (Laseron, 1956; McMichael, 1960). The behavior of a *Cerithium* here described may be one aspect of acclimation to drying conditions and perhaps of high temperature acclimation in a tropical intertidal gastropod (see recent review of Segal, 1961).

Clustering and dispersal in rhythm with the tides, of a population of the cerithiid, *Cerithium Clypeomorus moniliferum* Kiener, occurs on tropical Heron Island in the Capricorns off the Queensland coast of Australia. The observations recorded here were made between October, 1960, and January, 1962, principally in October through December, 1960, while the author was a Fulbright scholar in the Department of Zoology at the University of Queensland.

The ecology and distribution of intertidal organisms in relation to the geography and tides of Heron Island have been discussed by Endean *et al.* (1956). The Island is at present bordered on its southwestern and northeastern shores by formations of beachrock, a consolidated calcareous deposit of uncertain chemical origin (Revelle and Emery, 1957; Kaye, 1959); beachrock distribution at Heron Island has presented a changing picture over the last 125 years (Saville-Kent, 1893, pp. 94–95, 106–108; Steers, 1938).

BEHAVIOR OF CERITHIUM AT HERON ISLAND

The Heron Island population of *Cerithium* (Fosberg *et al.*, 1961) inhabited chiefly during October and November of 1960 a relatively smooth beachrock plateau on the western tip of the Island (Fig. 1). During high tide the animals were

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[1] Contribution No. 1265 from the Woods Hole Oceanographic Institution.

[2] This study was made while the author was a Fulbright scholar in the Department of Zoology at the University of Queensland during 1960–1961. His work was also supported by the Woods Hole Oceanographic Institution, by NSF Grant G-4403, and by a John Simon Guggenheim Memorial Fellowship, for all of which he would express deep appreciation. He is much indebted to the Great Barrier Reef Committee for use of the facilities of the Heron Island Marine Research Station and to the Department at the University of Queensland for its generous hospitality.

Dr. D. F. McMichael of the Australian Museum, Sydney, kindly identified the species of *Cerithium.*

Professor W. Stephenson of the University of Queensland and Miss Isobel Bennett of the University of Sydney indicated that they had earlier observed the behavior of *Cerithium* here described.

Mr. H. F. Manning, caretaker of the Heron Island Station, very kindly carried on observations on *Cerithium* distribution following the author's visit.

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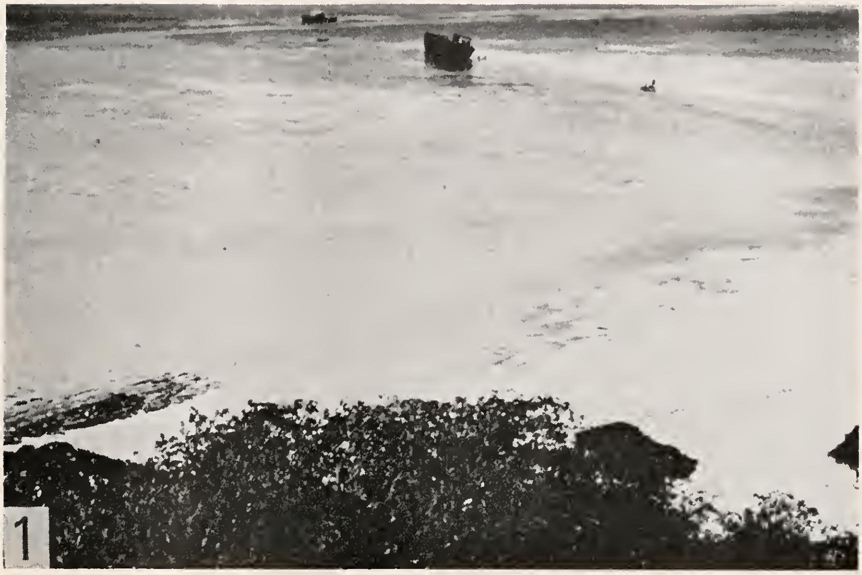

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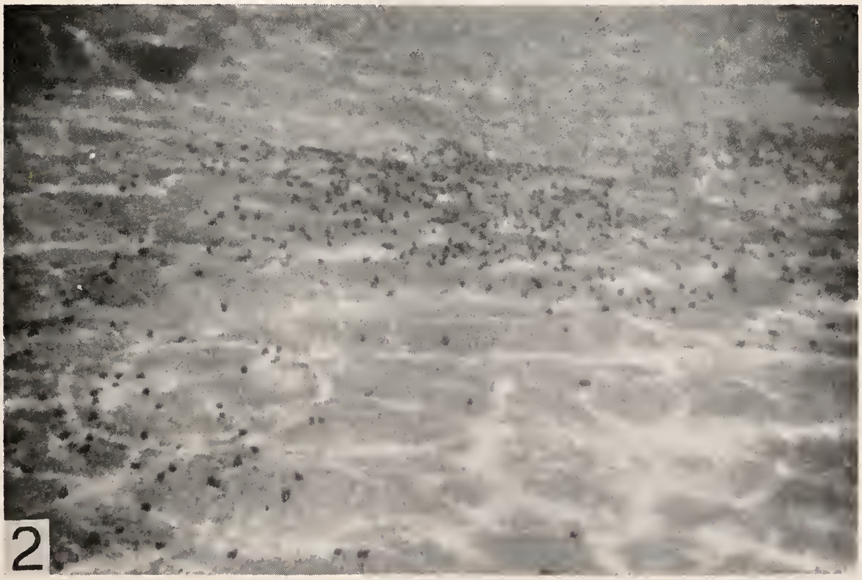


FIGURE 1. Appearance at low tide of the Heron Island *Cerithium* plateau (right center) on November 24, 1960. Algal scum had accumulated, graying most of the plateau.

FIGURE 2. A portion of the *Cerithium* population on the bottom at high tide on October 15, showing scattered distribution characteristic of tidal feeding. A clustering site of the preceding low tide is encircled in the left foreground.

homogeneously distributed over the bottom in the intertidal zone (Fig. 2); during low tide they were tightly clustered in roughly circular groups, generally of a few hundred to a few thousand individuals, on the open rock face (Fig. 3); an occasional isolated individual and clusters of a few to several snails occurred. *Cerithium* also clustered in sandy crevices and pits of rougher beachrock. Clustering occurred on the ebbing tide both night and day.

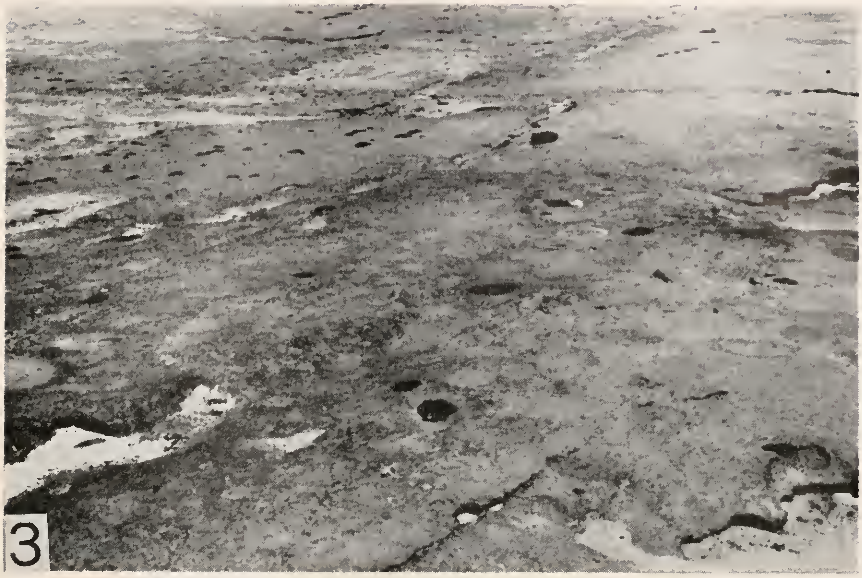
When on an incoming tide the water column attained 10 cm. over a *Cerithium* cluster, individuals began to move out from the edges of the cluster (Fig. 4). The clusters continued to disperse until the relatively homogeneous distribution of high tide over the feeding zone had been attained. On the ebbing tide, *Cerithium* began abruptly streaming toward various foci on the rock plateau under a water column of about 50 cm.; at 40 cm. the foci were clearly apparent with streams of converging *Cerithium* radiating around them; at 30 cm., the clusters were tightly formed (Fig. 5).

Observation of the behavior of marked individuals (shells marked with yellow enamel) of two clusters and of marked clustering sites in October, 1960, demonstrated that individual clusters were formed on successive tides of different components of the population, the painted individuals becoming increasingly dispersed during the period of observation; clustering sites varied on successive tides, there being no apparent predilection for specific sites.

Through November and December of 1960, the beachrock plateau occupied by the *Cerithium* population changed in character; a grey, silty scum of a blue-green alga (probably an *Oscillatoria*—R. Wood, personal communication) accumulated over the plateau. The *Cerithium* population became gradually restricted to sandy crevices and finally by mid-January, 1961, *Cerithium* had abandoned the plateau, and were beginning to concentrate at the southeastern end of Heron Island (Miss J. Badham, personal communication). Figures 3 and 6 contrast the appearance of similar areas of the plateau on October 17 when clustering was at its height and December 16, when the *Cerithium* population had become entirely restricted during low tide to sandy crevices in the plateau.

By October of 1961 the *Cerithium* population was concentrated in Shark Bay on the southeastern corner of Heron Island clustering and feeding over a sandy area above the nearby beachrock which at this time was apparently clear of algae (H. F. Manning, personal communication). Similar migrations have apparently occurred in the past; thus, Stephenson and Searles (1960) found a marked drop in the *Cerithium* population inhabiting experimental plots of beachrock near the Heron Island Marine Research Station between October 1, 1959, and January 12, 1960—a period corresponding to that of departure of the population from the western plateau in late 1960.

Clustering and dispersal occur experimentally in the absence of tidal rhythms. The single cluster removed to a shallow pan submerged in a pool of sea water behaves like a larger population; in shallow depths imitative of a falling tide, aggregation into a number of clusters occurs over a period of 24 minutes (Fig. 7). Subsequent lowering of the pan to a depth of 13 inches results in a gradual breaking up of the formed clusters as a search for food is initiated (Fig. 7). That is, changes in depth bring about distributions similar to those obtained over a normal tide cycle, independently of tidal rhythms. Tidal rhythms of physiological processes observed in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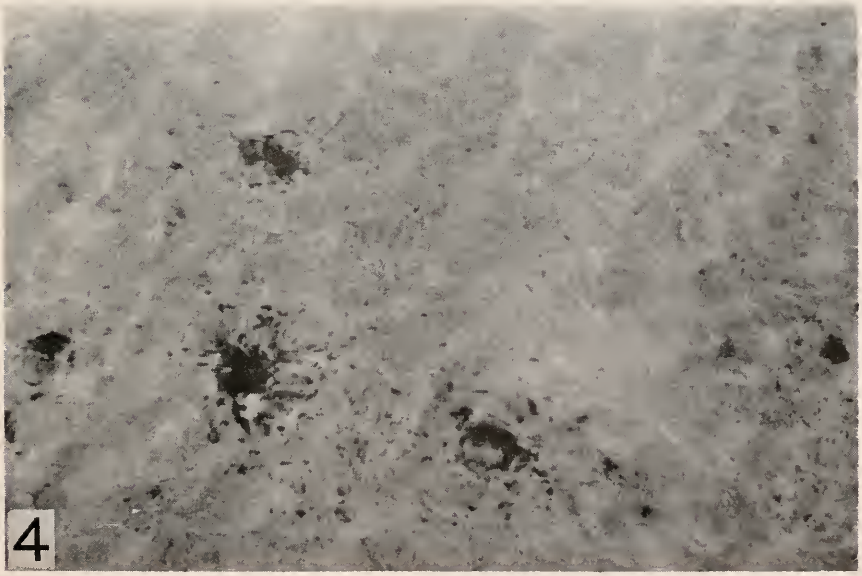
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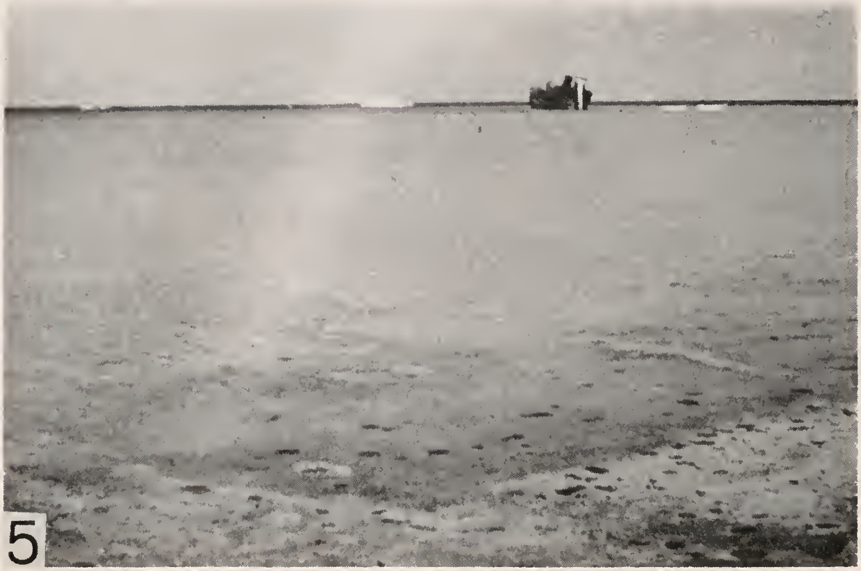
FIGURE 3. Clustered *Cerithium* exposed intertidally on October 15. Each black cluster contains from several to over a thousand individuals.

FIGURE 4. Individual *Cerithium* leaving clusters on the incoming tide on October 15.



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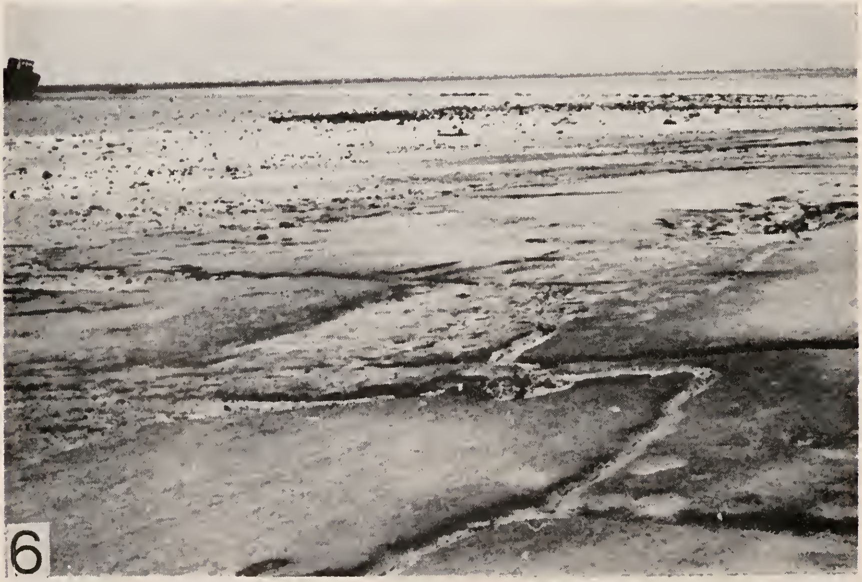

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FIGURE 5. Reaggregated and clustering *Cerithium* (dark patches) on a falling tide, still submerged, on October 15.

FIGURE 6. The *Cerithium* plateau in late November when *Cerithium* had retreated to sand-filled crevices, accompanying progressive accumulation of scum on the plateau. (Compare with Figure 3.)

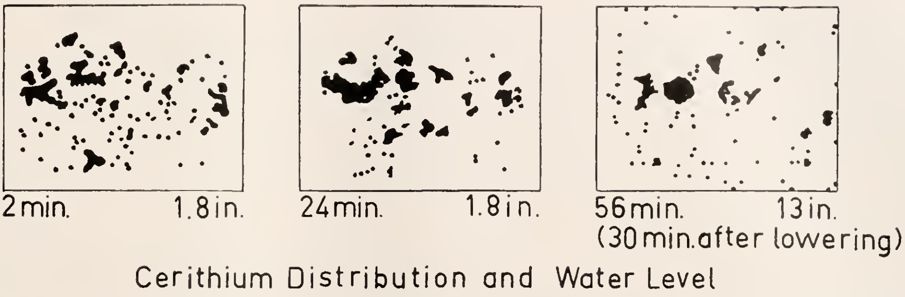

FIGURE 7. The distribution of a single cluster of *Cerithium* in a 12 inch × 14 inch enamel pan of sea water at various times and depths after immersion.

other mollusks (Rao, 1954) are, however, suggestive that *Cerithium* may respond in other ways to the tide cycle than by clustering and aggregation.

COMPOSITION OF THE POPULATION

The *Cerithium* population in late 1960 was comprised chiefly of two varieties of the same species: a dark gray translucently shelled form with a spire of lighter color than the major whorl, and a lighter colored, heavier shelled, less common form with a thickened lip similar in these details to the knobbed cerithium (*C. caeruleum*) of eastern Africa. Intermediates between the two types occur.

Size distribution of a cluster of 1377 individuals between 6 and 15.5 mm. in length is shown in Figure 8. Periodic recruitments to the population, presumably

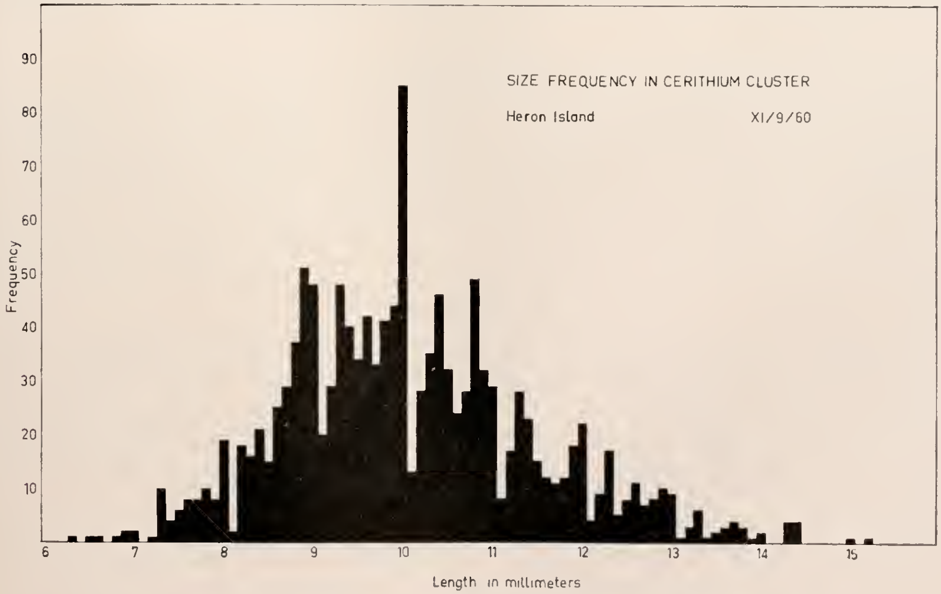

FIGURE 8. Size distribution within a single cluster of 1377 individuals collected on November 9, 1960.

of seasonal occurrence in view of their regular periodicity, are indicated by breaks in the spectrum of distribution at just above each millimeter mark.

In late 1960 there were no cerithiid egg filaments on the plateau (described by Ostergaard, 1950, for *Clava obeliscus*), nor did cerithiid veligers, described by Lebour (1944) for *C. ferrugineum,* occur in plankton towed in October and November in the channel between Heron Island and Wistari Reef; in fact veligers of any type were scarce. The smallest individual collected on the plateau during late 1960 measured 4 mm. in length.

Males bore active gametes, but ovaries were poorly developed and no ripe eggs were found in a few hundred females examined. Sex ratios were inconstant in clusters. In short, clustering seemed not related to reproductive activity. The reproductive habits of cerithiids vary considerably, as between species of *Cerithiopsis* (Lebour, 1933) and *Cerithium ferrugineum* (Lebour, 1944).

A variable proportion of the *Cerithium* population on the western plateau of Heron Island was parasitized by larval bird flukes, notably by a heterophysid opisthorchioid (Dr. John Pearson, personal communication) characterized by a cercaria with a large chocolate-brown tail; several other species occurred. Between November 18 and 23, 1960, 7% and 45% of small samples (60 each) of the thin- and thick-shelled forms, respectively, were parasitized, and in early January Miss J. Badham, an undergraduate of the University of Queensland, reported finding 23% of a random sample of 50 animals from the western part of Heron Island (where shore birds, especially the silver gulls, were most numerous) were infested with trematode larvae. By this time, a population of *Cerithium* had begun to accumulate at Shark Bay, and here only 1 to 2% of a sample of 30 were infested. Shore birds were of far less common occurrence in this area. Sindermann (1961) has reported that larval trematode infestation inhibits migrations of two north temperate gastropods.

Discussion

Whatever the factors that influence the choice of clustering site and clustering itself, it seems likely that the habit is an adaptation of *Cerithium* to drying conditions and high temperatures on tropical beaches, and that it falls into the habitat category of ecologically important homeostatic mechanisms suggested by Bullock (Segal, 1961). The clustering habit effectively decreases air temperature to which *Cerithium* is exposed at Heron Island at low tide during the daytime (30.6° C. on the sand surface; 27.8° in the center of a cluster on January 4, 1962—H. F. Manning, personal communication). However, water temperature may exceed air temperature by a degree or two over the clusters; and *L. neritoides* withstands temperatures up to 47° C. in air (Fraenkel, 1961). It seems likely that prevention of drying is a more significant result of clustering than is a lowering of temperature. It is suggested that clustering is a behavioral adaptation to tropical conditions, superimposed perhaps over physiological adaptations which have been demonstrated in other mollusks (Fraenkel, 1961; Segal, 1961).

Clustered individuals are always damp, and clusters taken to the laboratory, spread and allowed to dry during the first marking experiments, died rapidly. Within a cluster there is always some degree of movement of individuals, resulting in a slow turnover of the group. Further, a cluster contains a varying amount of damp sand or silt, and the shells of many individuals are decorated with small bits of green alga (probably an *Ulva*—R. Wood, personal communication). These factors combine to retain a moist environment for clustered *Cerithium* during low tide.

Consistent responses to height of the water column are suggestive that *Cerithium* possesses a hydrostatic mechanism which helps to determine whether the animals shall be clustered or dispersed. In behavioral terms one can envisage that the falling tide induces a positive barokinesis (increased rate of movement due to falling pressure) and clustering due to thigmotaxis (a search, induced by the falling tide, for contact surfaces which may be conducted along slime trails laid down by other animals, the search continuing until clustering results). According to this hypothesis, following the exposure period of low tide, barokinesis induced by an increasing water column or increased activity induced by the moisture of the incoming tide may induce the animals to terminate clustering in the search for food (see Carthy, 1958). A remarkable degree of sensitivity to the water column has been demonstrated in a crustacean (Enright, 1960), and there is some evidence that copepod sex ratios may be influenced by hydrostatic pressure (Vacquier, 1962).

Analogous aggregations of intertidal gastropods occur in northern waters with the onset of cold weather. Thus, in the Gulf of Maine and on the shores of Massachusetts' Cape Cod, populations of *Nassarius obsoletus* aggregate in dense concentrations (Jenner, 1958), probably preceding a general movement to deeper water from the intertidal mudflats (Sindermann, 1961). Rock face types such as the littorinids and *Thais* of the Maine coast have been observed to aggregate in clusters on the rock faces before moving into submerged positions in deep crevices; with the approach of warmer weather the movement is reversed as the animals reoccupy exposed situations. In all of these cases, however, the tidally rhythmic aggregations and dispersals demonstrated by *Cerithium* are lacking.

SUMMARY

1. A striking rhythmical clustering and dispersal of a population of *Cerithium Clypeomorus moniliferum* Kiener on Heron Island in the Capricorns of Australia is described.

2. A sequence of events preceding a seasonal migration of the concentrated *Cerithium* population is reconstructed.

3. The role of the described habit as an ecologically important homeostatic mechanism is discussed.

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THE LARVAL DEVELOPMENT OF CALCINUS TIBICEN (HERBST) (CRUSTACEA, ANOMURA) IN THE LABORATORY[1]

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Since the first studies on hermit crab development in the early 19th century, workers have had to rely on reconstructions of developmental sequences from planktonic material. In some instances it has been possible to maintain larvae taken from the plankton through one moult in the laboratory and thus to tie together series of larvae believed to represent single species. This was the method used by Thompson (1904) in his classic work on *Pagurus* development, and by more recent workers as well (MacDonald, Pike and Williamson, 1957; Pike and Williamson, 1960; Dechancé, 1961). The reconstruction method may be used profitably only in waters with restricted faunas. Planktonic larvae from any but the most intensively studied areas can seldom be identified with certainty. In tropical waters which are generally less well studied and in which there are usually several times the number of species and genera found in temperate seas, the reconstruction technique is much less useful.

Although the tropical Western Atlantic contains the second richest pagurid fauna in the world (Wass, unpubl.; Provenzano, 1959, 1961a, 1961b) there is not a single species of the family Diogenidae in the West Indies for which the larval development is known. Excepting two species of *Paguristes* which have abbreviated development (Hart, 1937; Pike and Williamson, 1960) no hermit crabs of that family have previously been reared in the laboratory from the first zoea to the post-larval stage.

Fortunately, in recent years advances have been made in the development of techniques for rearing several decapod groups in the laboratory. Broad (1957a, 1957b) and Dobkin (in press) have been able to rear caridean shrimps. Costlow and Bookhout (1959, 1960a, 1960b, 1961a, 1961b), Knudsen (1958, 1959a, 1959b), Hart (1960) and Chamberlain (1961) have been successful with a variety of brachyuran crabs. Forss and Coffin (1960) pointed out the applicability of a method for rearing several decapod groups and Coffin (1960) was able to rear a species of the hermit crab family Paguridae. At this laboratory a terrestrial hermit crab of the family Coenobitidae has been successfully reared to metamorphosis (Provenzano, in press).

Until very recently there have been no descriptions of larvae of the tropical hermit crab genus *Calcinus*. According to Pike and Williamson (1960), the glaucothoe stage described and figured by Bouvier (1922) and attributed by him to *Clibanarius* may be the postlarva of *Calcinus ornatus* (Roux) of the Mediterranean. Bourdillon-Casanova (1960) described and figured a zoea taken from the plankton, which she believed to be the first stage of *Calcinus ornatus*. Pike and Williamson (1960) were able to obtain the first zoea of *C. ornatus* from a laboratory hatching and by holding identical larvae from the plankton through one moult in the laboratory they were able to obtain the second stage. Similarly they obtained and described five zoeal stages and one glaucothoe stage for that species.

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In the Western Atlantic only two species of *Calcinus* are known to occur (Provenzano, 1960). Lewis (1960) gave a very brief description and a figure for the first zoea of *Calcinus tibicen* (Herbst) but neither is sufficiently detailed for use in identification. No other reports on larvae of this genus are known. The present paper describes the larval development of *Calcinus tibicen* in the laboratory.

Materials and Methods

Ovigerous females of *Calcinus tibicen* were collected at Bear Cut, Biscayne Bay, Florida. In the laboratory they were kept in large fingerbowls containing filtered sea water until hatching occurred. In the first experiment, conducted from 7 June 1960 to 3 August 1960, approximately 40 larvae were removed by means of a wide-bore pipette to plastic compartmented trays, five larvae to each compartment of about 50 cc. capacity. Every second day larvae were transferred to corresponding compartments in fresh trays containing filtered sea water and freshly hatched *Artemia* nauplii as food. Temperature of the standing sea water during this period gradually increased from 26.5° C. to 30.3° C. with diurnal fluctuations of about 1° C. Salinity samples taken at each change of water were titrated and values ranged from 31.1 ppt to 35.0 ppt. The second experiment ran from 17 April 1961 to 7 June 1961. Approximately 150 larvae were placed singly in individual compartments containing filtered sea water and freshly hatched *Artemia*. A number was assigned to each compartment and every second day larvae were transferred to correspondingly numbered compartments in freshly prepared trays. By daily examination of the trays for exuviae and for dead animals, and by preserving exuviae of each larva in an appropriately numbered vial it was possible to follow the complete history of each specimen. Temperatures ranged from 26.0° C. to 29.0° C. during the experiment. Salinity was semi-controlled by storage of filtered sea water in five-gallon carboys. Three lots of water were used during the development of these larvae, according to the following schedules: from 17 April to 12 May, salinity of 35.52 ppt during stages I–V; from 12 May to 22 May, salinity of 37.41 ppt during stages V, VI, some VII, and glaucothoe; and from 22 May to termination of the experiment, salinity was 37.07 ppt and affected stages VII, VIII, and some glaucothoes. No attempt was made to control illumination other than to prevent direct sunlight from falling onto the transparent boxes.

Larvae and exuviae were preserved in 5–7% sea water-formalin buffered with hexamethylene tetramine or were rinsed in fresh water and stored in 70% ethyl alcohol. After staining in Mallory's acid fuchsin red, specimens and exuviae were dissected in 85% lactic acid and appendages were mounted in Hoyer's medium. Drawings of whole larvae were made from live, immobilized animals or from freshly killed specimens. A stereoscopic dissecting microscope with magnifications up to 36 × was used for dissections and for making camera lucida drawings of whole animals. Enlargements of appendages and other parts were drawn using a camera lucida with a monocular microscope at 90 × and details were checked at 450 ×.

The descriptive portion of the text was based chiefly on specimens under the microscope at the time of description. Usually appendages of at least two or three, sometimes five or six, different animals were examined during preparation of the descriptions. Notes made on additional material were incorporated later.

Setae were drawn as they appeared with respect to number, position, and length but setules when present were illustrated semi-diagrammatically. In most illustrations of appendages the setules are shown as somewhat shorter and much less numerous than in the specimens.

Duration refers to the time range between moults for animals which successfully passed through a given stage. This "normal" duration was often exceeded by larvae which died without moulting to the subsequent stage and such data are not included in the values for duration.

Measurements were made with the aid of an ocular micrometer. Total length (TL) was measured from the tip of the rostrum to the posterior border of the telson, exclusive of all telson processes. Length of carapace (CL) was measured from the tip of the rostrum to the most posterior lateral margin of the carapace including the lateral spines. The numbering of telson processes follows the system of Pike and Williamson (1960) not MacDonald, Pike and Williamson (1957).

The females from which larvae were obtained are on deposit in the Marine Museum of the Institute of Marine Science, University of Miami.

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RESULTS

The number of zoeal stages through which *Calcinus tibicen* may pass in the laboratory before moulting to the glaucothoe stage is variable. The glaucothoe was obtained after six, seven or eight zoeal instars. The principal features of each stage are as follows:

First Zoea. Figs. 1, I; 2, I.
Size. TL 1.9 mm.; CL 1.3 mm.
Duration. From five to eight days.

The carapace has a prominent carinate rostrum and there is a large corneous submarginal spine postero-laterally on each side of the carapace. The eyes are immobile. The fifth abdominal somite has a prominent very slightly curved medio-dorsal spine and a smaller lateral spine on each side of the same somite. There is a dorso-lateral pair of fine hairs on somites two, three and four which are not noticed on later stages. The sixth somite is fused to the telson (Fig. 3, I) which is broader than long, notched medially, and armed with seven processes on each side. The first or outermost process is apparently fused to the telson, but a line of demarcation may be seen. The second process is a fine hair bearing setules. Processes three to seven are strong plumose spines articulated with the telson. There is a red pigment

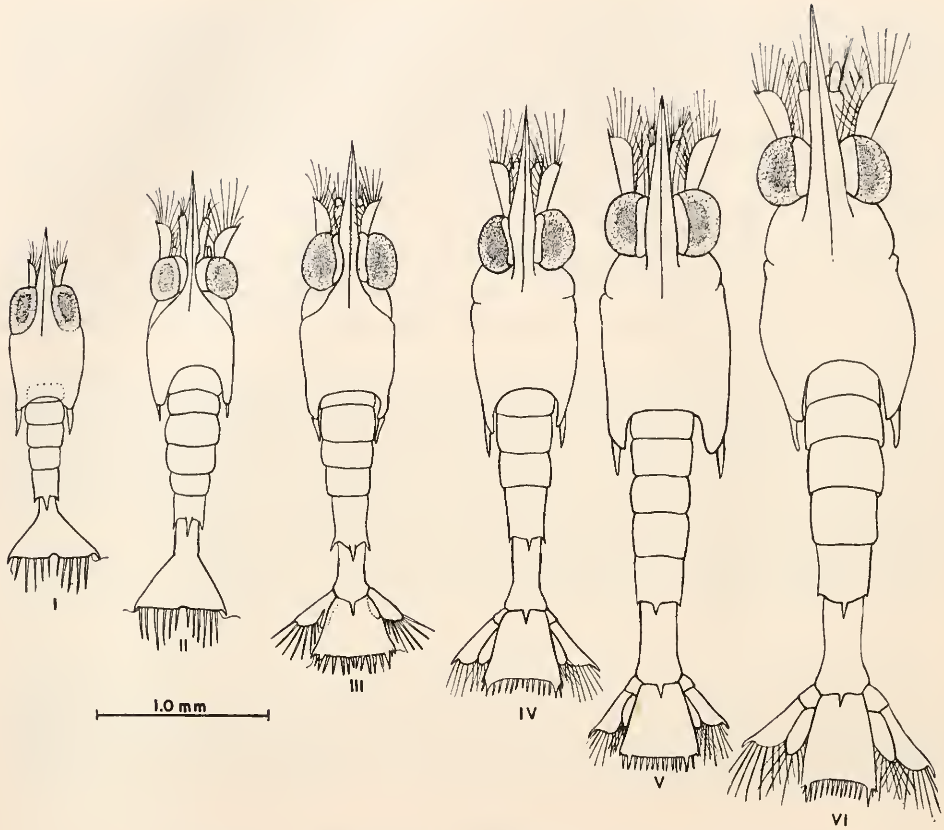

FIGURE 1. *Calcinus tibicen.* Zoeal stages I–VI, dorsal view.

spot at about the mid-point of the antennule and diffuse orange-red pigment over the thorax, but under the carapace. In some specimens there is a diffuse bluish pigment on the telson.

The antennule (Fig. 4, I) terminates in at least one aesthete and four other processes. There is a very prominent subterminal plumose seta.

The antennal endopodite is fused to the basipodite and bears two long terminal plumose setae and one subterminal seta less than one-half as long as the others (Fig. 5, I). A short, toothed spine is present on the ventral surface of the basipodite. The antennal scale is about three times longer than wide, slightly longer than the endopodite, is concave on the lateral margin and bears a terminal tooth. Subterminally there is a short hair followed by nine, rarely eight, longer plumose setae.

The mandibles are dissimilar, simple toothed processes.

The three-segmented endopodite of the maxillule (Fig. 6, I) has three terminal setae with another distally on the second segment. The proximal endite of the basipodite has six setae of which four are branched, two simple. The distal endite terminates in two large spines and two non-plumose setae.

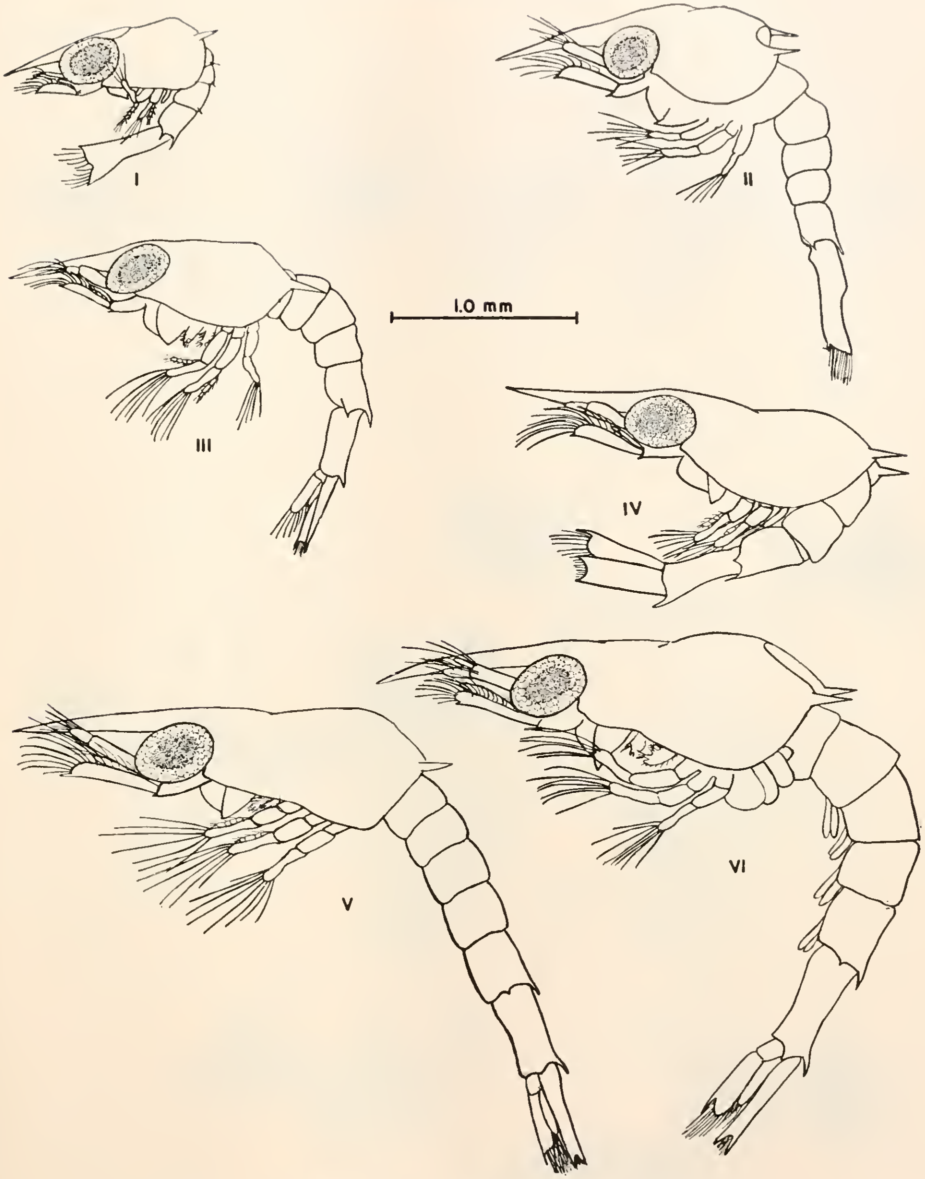

FIGURE 2. *Calcinus tibicen.* Zoeal stages I–VI, lateral view.

The maxilla (Fig. 7, I) bears six or seven setae on the proximal lobe of the coxal endite, four on the distal lobe. There are four setae on both proximal and distal lobes on the basal endite. The bilobed unsegmented endopodite bears four setae, the scaphognathite five plumose setae.

The first maxilliped (Fig. 8, I) has a prominent curved seta upon a papilla

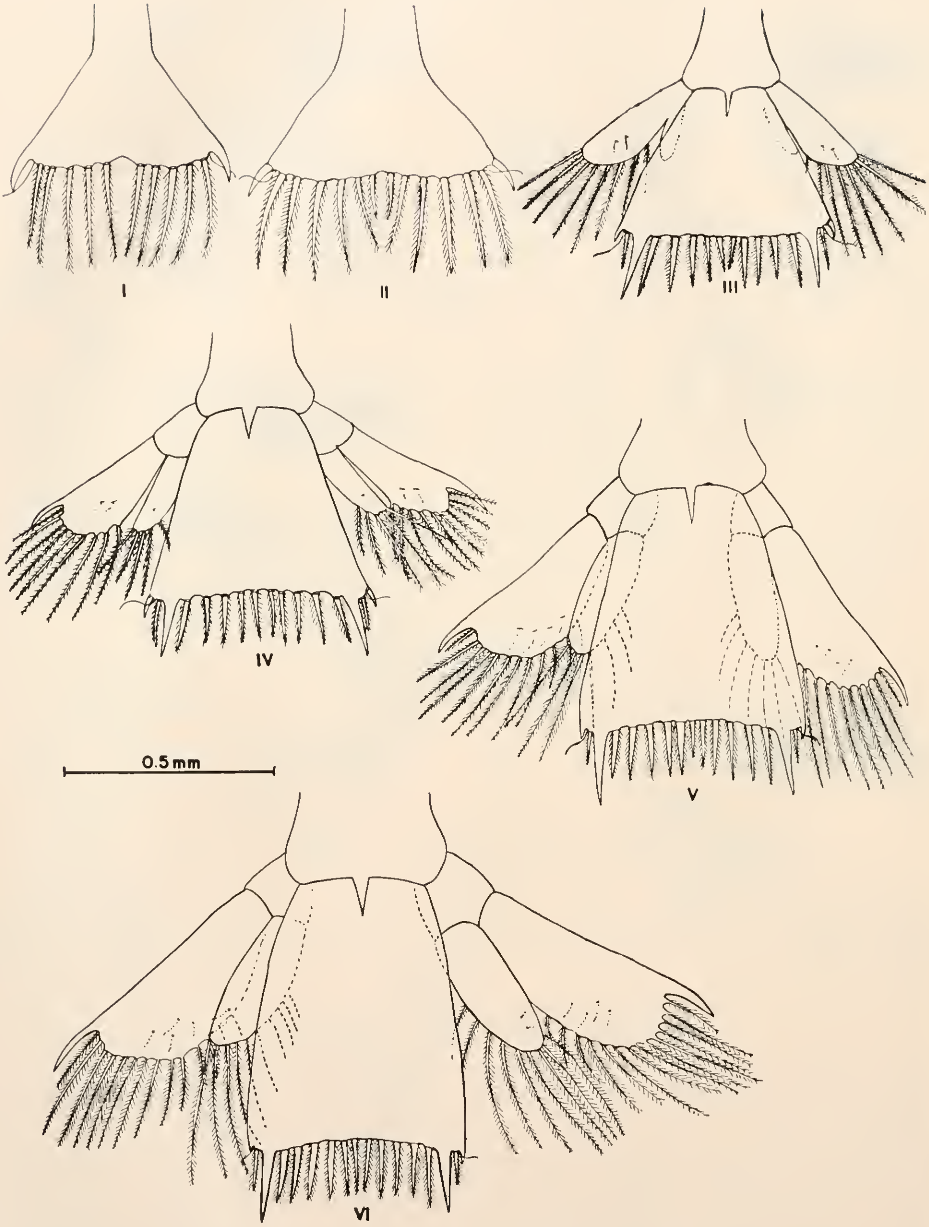

FIGURE 3. *Calcinus tibicen.* The telson, zoea I–VI.

proximally on the medial margin of the basipodite, with about nine additional setae along the medial margin. The exopodite is composed of two indistinct segments and terminates in four long plumose setae. The endopodite is five-segmented, the terminal segment bearing four plumose setae apically with a fifth proximally. The

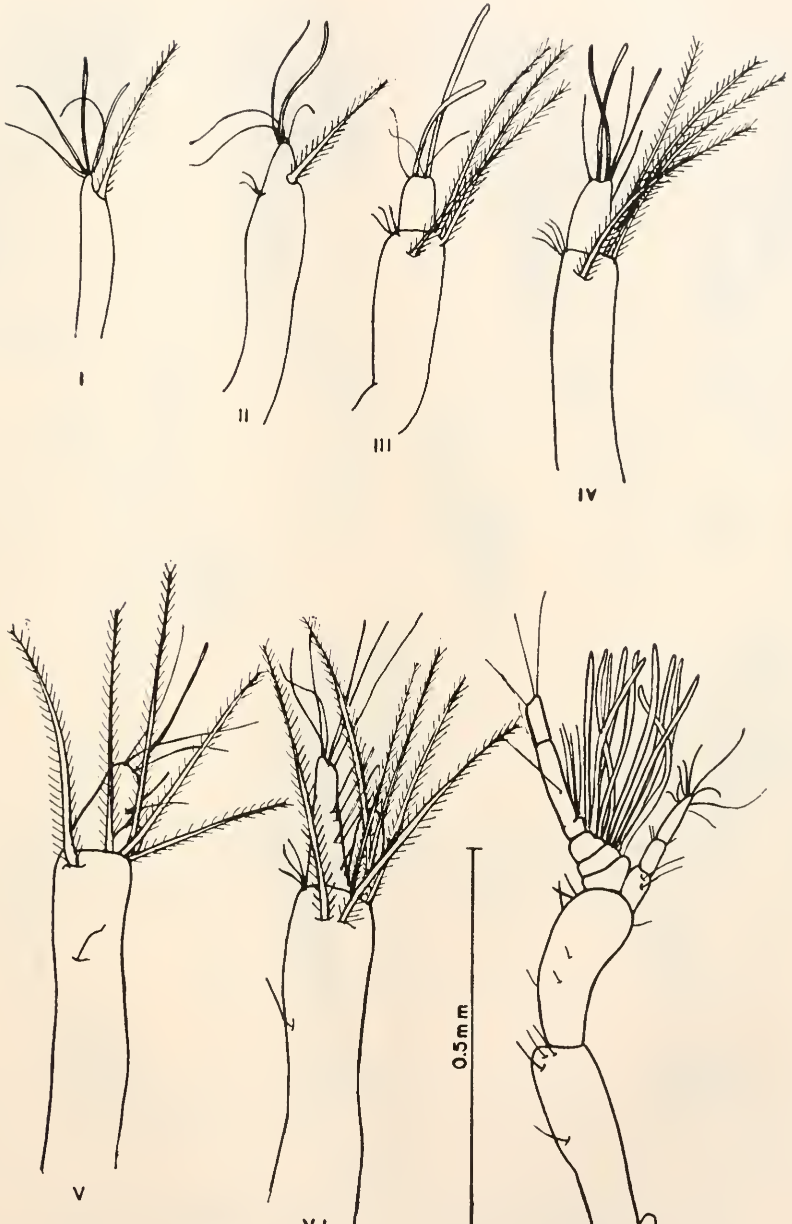
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FIGURE 4. *Calcinus tibicen.* The antennule, zoea I through glaucothoe.

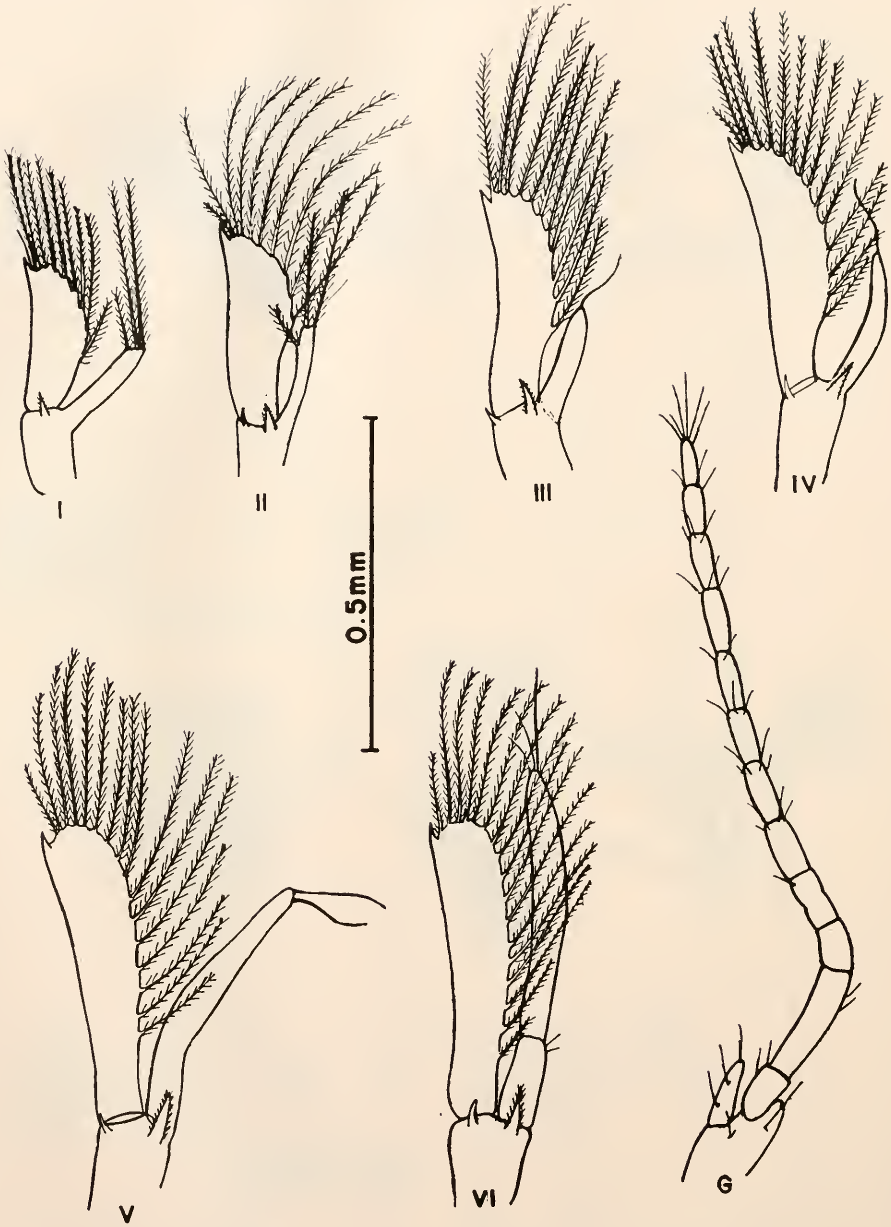

FIGURE 5. *Calcinus tibicen.* The antenna, zoea I through glaucothoe.

antepenultimate segment bears a single distal hair medially. The other segments bear a pair of setae medially. There is a row of very fine setules along the lateral margin of the endopodite.

The second maxilliped (Fig. 9, I) has only three setae on the medial margin of

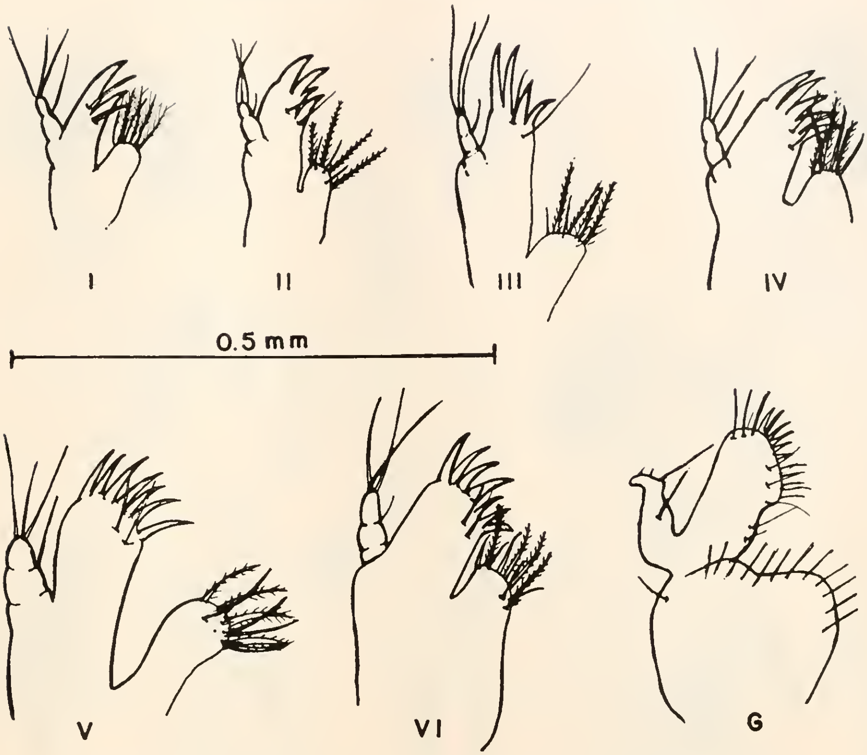

Figure 6. *Calcinus tibicen.* The maxillule, zoea I through glaucothoe.

the basipodite. The exopodite has four long plumose terminal setae. The endopodite is only four-segmented, the terminal segment having four apical setae and one seta subdistally. The other segments each have a pair of distal setae medially.

The third maxilliped (Fig. 10, I) is a uniramous rudiment.

Second Zoea. Figs. 1, II; 2, II.
Size. TL 2.5–2.6 mm.; CL 1.5 mm.
Duration. From three to five days.

Mobile eyes serve to distinguish the second zoea from the first. The dorsal spine of the fifth abdominal somite is still very prominent, much larger than the laterals. The sixth somite is still fused to the telson which bears an extra pair of processes medially (Fig. 3, II). The outermost process on each side of the telson is clearly not fused to the telson. In addition to the same red-orange coloration as noted for the previous stage, there is diffuse blue on the anterior carapace ventrally and a blue cast to the carapace dorsally, especially in the cardiac region.

The antennule (Fig. 4, II) has five or six terminal processes, at least one of which is an aesthete. There is a large plumose seta subterminally and a pair of small fine setae at about the same distance but on the opposite side.

The antenna (Fig. 5, II) is essentially unchanged in form and setation. There

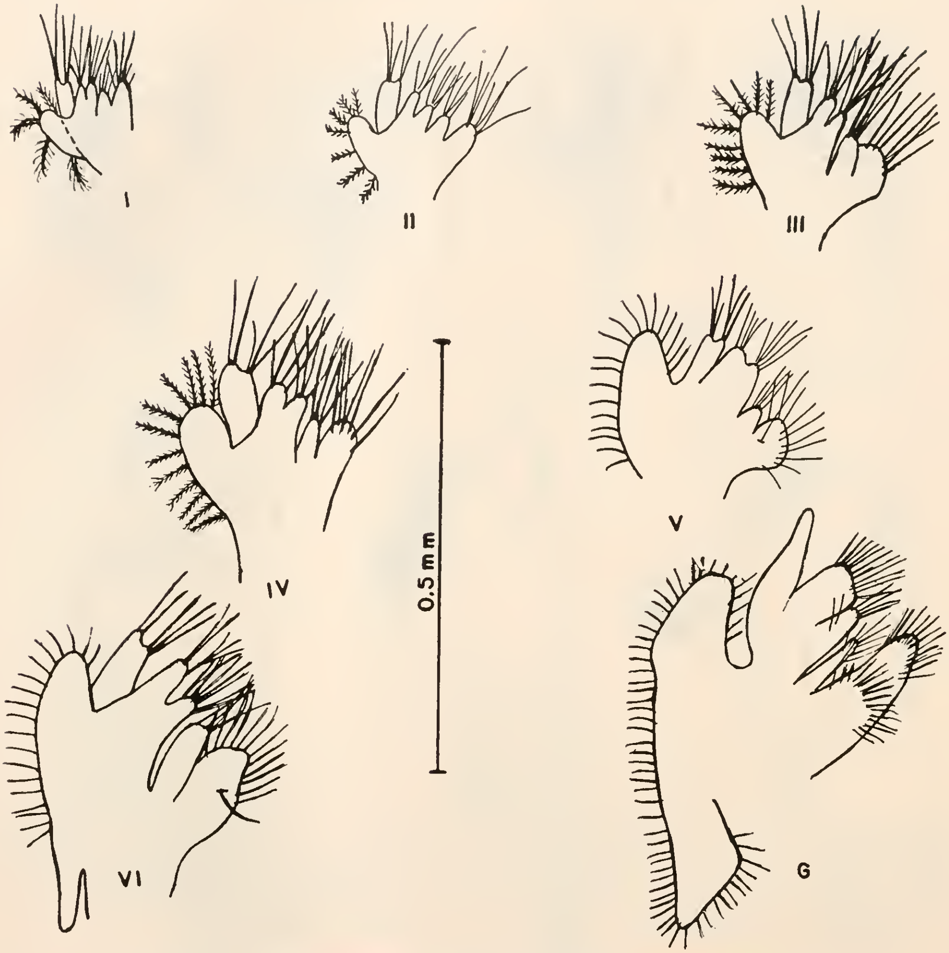

FIGURE 7. *Calcinus tibicen.* The maxilla, zoea I through glaucothoe.

is a small tooth laterally on the basal portion in addition to the spine at the base of the endopodite.

The mandible is unchanged to any notable degree.

The segmented endopodite of the maxillule (Fig. 6, II) is unchanged and the coxal endite still has four feathered setae and three simple setae but the basal endite now has four large teeth and two setae.

The maxilla (Fig. 7, II) has seven setae upon the proximal lobe of the coxal endite and four setae on the distal lobe. Apparently there are five on the proximal lobe of the basal endite and four on the distal lobe. The endopodite has two proximal and three distal setae. The scaphognathite has six or seven short plumose setae. The first maxilliped (Fig. 8, II) has six plumose natatory setae on the exopodite. The endopodite still has five setae on the terminal segment and there are two distally

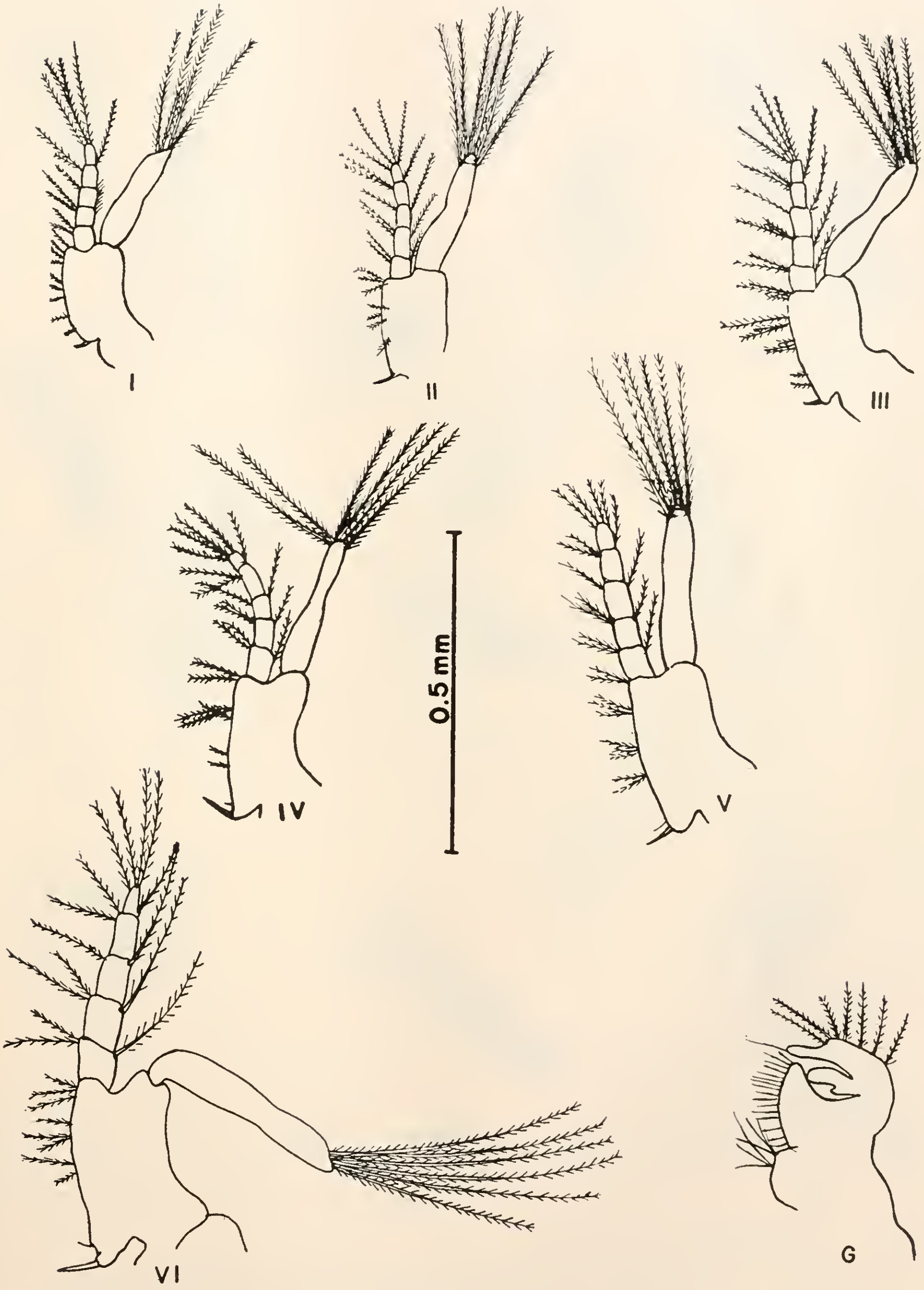
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FIGURE 8. *Calcinus tibicen.* The first maxilliped, zoea I through glaucothoe.

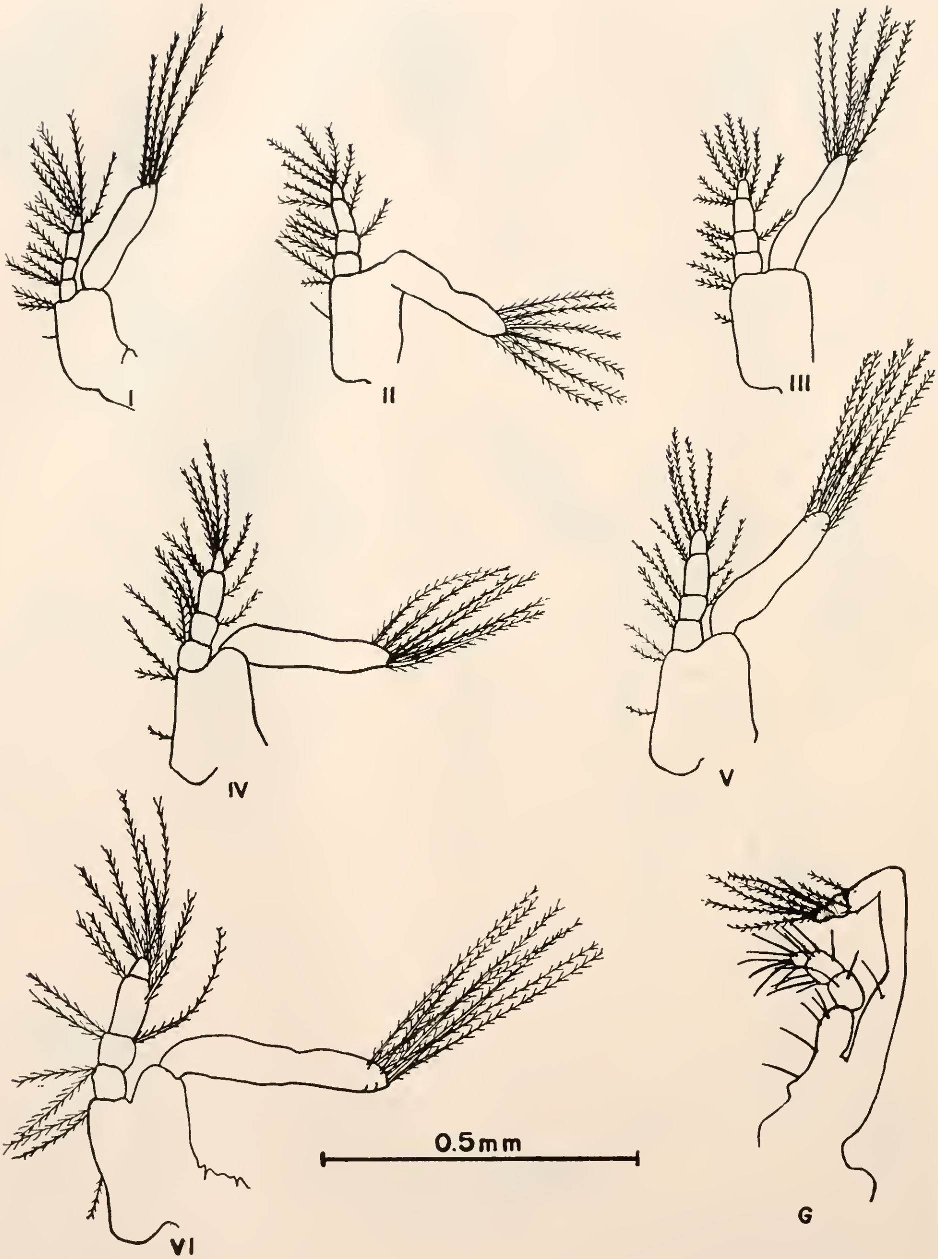
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Figure 9. *Calcinus tibicen.* The second maxilliped, zoea I through glaucothoe.

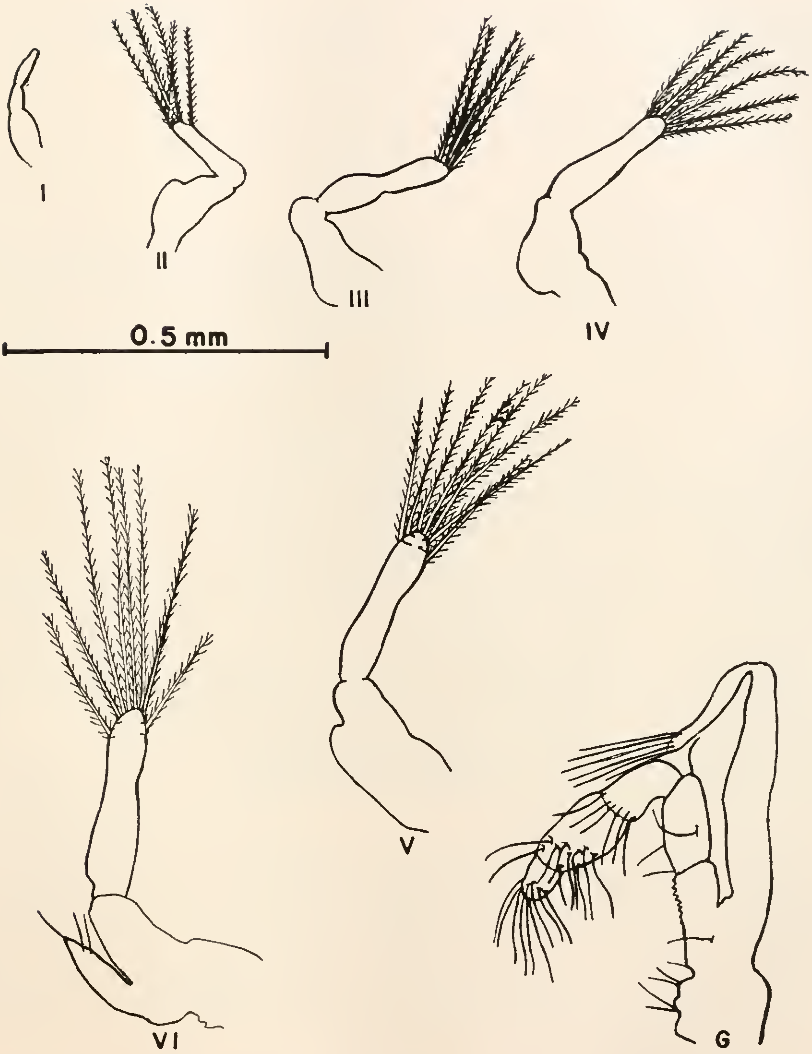

FIGURE 10. *Calcinus tibicen.* The third maxilliped, zoea I through glaucothoe.

on the medial side of the penultimate segment. The antepenultimate segment bears one seta medially and one laterally. The two proximal segments each bear one seta laterally and two medially. There are eight or nine setae along the medial margin of the basipodite in addition to the proximal hooked process.

The second maxilliped (Fig. 9, II) has six natatory setae on the exopodite. The four-segmented endopodite bears five setae on the terminal segment and on each of the two next proximal segments there are three setae, one of them laterally, two medially. The most proximal segment has only two setae medially. There are three setae on the medial margin of the basipodite.

The third maxilliped (Fig. 10, II) is a jointed uniramous appendage with five plumose terminal setae.

Third Zoea. Figs. 1, III; 2, III.
Size. TL 3.0 mm.; CL 1.7–1.8 mm.
Duration. About five days, from approximately the 12th day after hatching to the 17th day.

Aside from increase in size there is no major change in form of the cephalothorax. The fifth abdominal somite still bears a prominent medio-dorsal spine and a pair of lateral spines. The sixth abdominal somite is no longer fused with the telson and bears a postero-medio-dorsal spine as well as unjointed uropods (Fig. 3, III). The exopodite of the uropod bears usually eight, sometimes seven, plumose setae and has two inconspicuous hairs situated medio-ventrally while the endopodite of the uropod is a simple unarmed bud. The telson is armed laterally with an unfused hairless tooth. The second process as in earlier stages is an inconspicuously plumose hair and medially to this third process is a feathered articulated spine. The fourth process has become a large unarmed fused spine. Processes five to nine are articulated plumose setae. The total count of telson processes is 9 + 9.

The antennule (Fig. 4, III) is segmented. The terminal segment bears two large and three fine processes while the basal peduncle bears three large plumose setae and two or three very small setules.

The antennal scale (Fig. 5, III) bears about 12 medial plumose setae. The articulated endopodite has a single terminal seta. The armed spine and the hooked tooth at the base of the endopodite and scale are still present.

The mandible has a few more very small teeth on the cutting edge.

The maxillule (Fig. 6, III) is essentially unchanged.

The maxilla (Fig. 7, III) appears unchanged except for the scaphognathite which may have up to 11 plumose setae.

The first maxilliped (Fig. 8, III) has six natatory setae on the exopodite. The five-segmented endopodite has four terminal setae and one proximal seta on the ultimate segment. The penultimate segment bears a pair of setae medially. The antepenultimate segment bears one medial and one lateral, while the next two proximal segments each bear one seta laterally and one large and one small medially. There are three groups of about three setae on the medial margin of the basipodite and two setae near the proximal hooked process.

The second maxilliped (Fig. 9, III) also has six natatory setae on the exopodite. The four-segmented endopodite has on the distal segment four terminal setae and one proximal seta. The penultimate segment bears two setae medially, one laterally as does the antepenultimate segment, but the first segment bears only two medial setae. There are three or four setae on the medial margin of the basipodite.

The third maxilliped (Fig. 10, III) bears five or six natatory setae.

Fourth Zoea. Figs. 1, IV; 2, IV.
Size. TL 3.8–4.2 mm.; CL 2.0–2.4 mm.
Duration. Usually four or five days, rarely six.

The spines of the fifth abdominal somite have become relatively smaller, especially the lateral. The telson armature (Fig. 3, IV) may be similar to that of the preceding stage but in six of seven specimens taken at random and examined, a very small medial articulated spine was also present. The most readily noted characteristic of this stage is the changed armature of the now articulated uropods. The exopodite of the uropod has a strong curved outer spine with nine or ten plumose setae along the inner margin of the uropod. The endopodite bears four or five plumose setae. The exopodite has two medio-ventrally placed very inconspicuous hairs; the endopodite has one.

The antennule (Fig. 4, IV) has its terminal segment ending in two large and four small sensory processes. At the distal end of the proximal segment there are four small setules and four large plumose setae.

The antenna (Fig. 5, IV) is basically unchanged but now there are 13–15 setae in addition to the terminal tooth on the antennal scale. The endopodite still terminates in a single seta.

The mandible shows no essential change.

The maxillule (Fig. 6, IV) has on the coxal endite three or four curved plumose setae and a similar number of simple setae. The basal endite has usually five but occasionally six strong teeth plus two setae. The endopodite is unchanged.

The maxilla (Fig. 7, IV) has from 9–12 plumose setae on the scaphognathite. The endopodite bears four large setae and one smaller. The proximal lobe of the coxal endite bears seven to eight setae; the distal lobe, four. The proximal lobe of the basal endite bears four; the distal lobe also four.

The first maxilliped (Fig. 8, IV) still bears a prominent curved seta at the proximal medial corner of the basipodite and distally there are two, three and two setae along that margin. The exopodite bears six natatory setae. The five-segmented endopodite has five setae on the terminal segment, a pair distally on the penultimate segment, and one laterally, one medially on the antepenultimate segment. There are two medial and one lateral setae distally on the next segment and again on the most proximal segment.

The second maxilliped (Fig. 9, IV) also bears six natatory setae on the exopodite. On the four-segmented endopodite there are five setae terminally, two medially and one laterally on both the penultimate and antepenultimate segments and only two medial setae on the proximal segment.

The third maxilliped (Fig. 10, IV) is a jointed uniramous appendage with sometimes four, more usually five or six natatory setae.

Fifth Zoea. Figs. 1, V; 2, V.
Size. TL 4.3–4.9 mm.; CL 2.3–2.7 mm.
Duration. Usually five days, occasionally four or six.

This stage differs little in gross appearance from the preceding stage, but the length of the telson is now about equal to the width and there is an articulated medial telson spine in most specimens, making the armature formula 9 + 1 + 9 (Fig. 3, V). The exopodite of the uropod retains the curved outer spine and now bears 10–11 plumose setae while the endopodite bears from six to seven. The exopodite bears four inconspicuous setae ventrally.

The antennule (Fig. 4, V) has about four terminal sensory aesthetes. There are usually three, rarely four large plumose setae distally on the basal segment and another large seta with three or four very fine processes subdistally. A simple lobe arises from the same distal area of the basal segment or peduncle.

The antenna (Fig. 5, V) is basically unchanged, the scale bearing 14–16 plumose setae. The endopodite reaches two-thirds the length of the scale and terminates with one or two processes.

The mandible is essentially unchanged, having added a few fine teeth.

The maxillule (Fig. 6, V) has four curved plumose setae on the proximal endite and four simple setae. On the distal endite there are five to seven strong teeth and two setae. The endopodite is unchanged.

The maxilla (Fig. 7, V) bears 13–19 plumose setae on the scaphognathite. The endopodite bears three plus three setae. The distal lobe of the basal endite bears four setae, the proximal lobe five; the distal lobe of the coxal endite bears four, the proximal lobe 9–11 setae.

TABLE I

Characters of a "terminal" zoea VI and a "non-terminal" zoea VI

	Larva no. 49	Larva no. 117
Leg buds	very well developed	very small
Pleopod buds	present	not present
Telson armature	9 + 1 + 9	10 + 10
Telson L/W ratio	2.4/1.8	2.2/1.7
Uropod setae		
exopodite	13–14	12
endopodite	9	7

The first maxilliped (Fig. 8, V) has groups of about two, three, three and two setae on the medial margin in addition to the curved proximal process and a single seta near it. The exopodite bears six natatory setae, of which four are situated on a partially distinct distal segment. The terminal segment of the endopodite bears five setae. The penultimate segment bears two medial setae distally. The antepenultimate segment bears one seta medially and another laterally. The two proximal segments each bear two medial setae and one lateral seta.

The second maxilliped (Fig. 9, V) has three setae on the medial margin of the basipodite. The exopodite bears six or seven natatory setae. The endopodite is similar to that of the preceding stage.

The third maxilliped (Fig. 10, V) is uniramous and bears seven or eight natatory setae. There may be the beginning of an endopodal lobe.

Sixth Zoea. Figs. 1, VI; 2, VI.
Size. TL 4.8–5.6 mm.; CL 2.6–3.3 mm.
Duration. Usually four or five, rarely six days.

Larvae in the sixth zoeal stage may produce a glaucothoe directly or may moult into a seventh zoeal stage. The degree of apparent difference between stage VI and stage V or between two stage VI larvae is related to the future fate of the larva. For example, some characters of two stage VI larvae which died without moulting are shown in Table I.

Larva no. 49, had it survived, probably would have produced a glaucothoe, judging from results with other larvae. Larva no. 117 would have produced another zoeal stage, the form of which was distinguishable beneath the cuticle. In the present study three classes of stage VI larvae were observed but were not always distinguished before termination of the experiment. Some moulted directly from stage VI to the glaucothoe; some moulted from VI to VII and then to glaucothoe; some moulted from VI to VII to VIII and then to glaucothoe. The following description of stage VI is based primarily on a series (no. 65) in which the glaucothoe was produced directly from this stage.

The telson (Fig. 3, VI) is distinctly longer than wide.

The antennule (Fig. 4, VI) bears three large and several smaller terminal aesthetes. There are three subterminal pairs of processes. The lobe at the joint of the terminal and basal segments extends nearly to the end of the terminal segment. There are three small setae and four large plumose setae at the distal end of the basal segment, with another large one (or two in some specimens) more proximally.

TABLE II

Characters of several stages in a series producing the glaucothoe after six zoeal stages.
(Larva no. 65)

Stage	III	IV	V	VI
Telson L/W	1.4/2.0	1.8/2.0	2.1/2.0	2.4/2.0
Telson armature	9 + 9	9 + 9	9 + 1 + 9	9 + 1 + 9
Uropod setae				
exopodite	8	10	12	14
endopodite	0	5	7	9

The antennal scale (Fig. 5, VI) bears 16 or 17 plumose setae while the endopodite which is now two-segmented exceeds the scale in length and terminates in a single hair (but occasionally in three, of which two are small and inconspicuous).

The mandible is not changed notably. The bud of a mandibular palp could not be distinguished in available material.

The maxillule (Fig. 6, VI) is unchanged with respect to the endopodite but the basal endite has five apparently articulated and two non-articulated teeth and two simple setae. The coxal endite bears four larger curved plumose setae and five smaller simple setae.

The maxilla (Fig. 7, VI) upon the proximal lobe of the coxal endite bears 13 setae, on the distal lobe four. The proximal lobe of the basal endite bears eight, the distal lobe four or five setae. The endopodite bears five setae and the scaphognathite has about 19–20 plumose setae.

The first maxilliped (Fig. 8, VI) still bears the curved proximal process on the basipodite with additional setae along the medial margin. The exopodite bears six natatory setae. The five-segmented endopodite has the same arrangement of setae as in the preceding stage.

The second maxilliped (Fig. 9, VI) bears three setae on the medial margin of the basipodite and the exopodite bears seven natatory setae. The armature of the four-segmented endopodite is similar to that of the preceding stage.

The third maxilliped (Fig. 10, VI) may be uniramous in a long developmental sequence. When the sixth zoea is the penultimate zoea in a series the basipodite may be slightly swollen. When it is the last zoea, the third maxilliped may be biramous, the endopodite being represented by a long lobe arising from the proximal

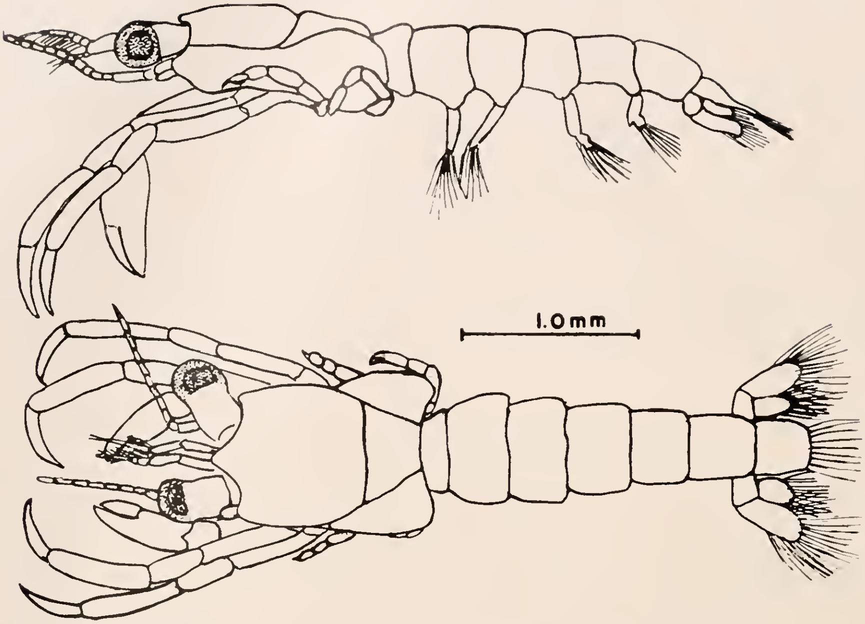

FIGURE 11. *Calcinus tibicen.* The glaucothoe, lateral view (above) and dorsal view (below).

portion of the basipodite and bearing two or three setae. The exopodite in a terminal stage VI usually bears eight natatory setae.

Zoeal stages VII and VIII.

These stages, both or the first of which were sometimes passed through prior to attainment of the glaucothoe, are the result of non-uniform rates of internal growth coupled with a more or less regular moulting cycle. There were no essential additions or changes from preceding stages except that where the preceding stages were somewhat retarded as regards degree of setation, these later stages appeared to make

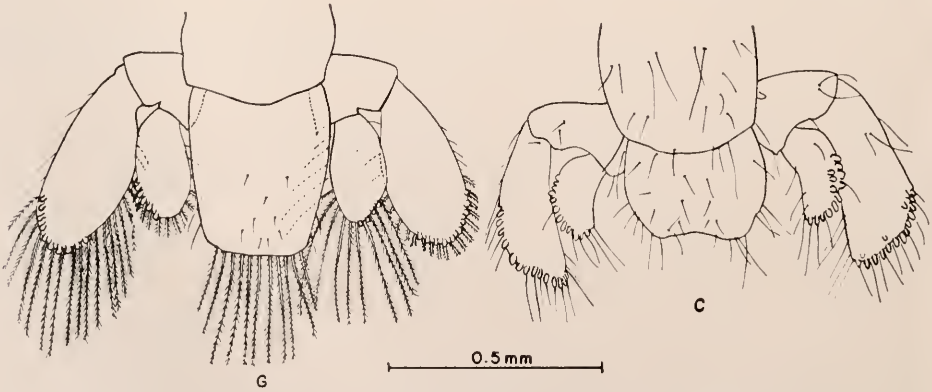

FIGURE 12. *Calcinus tibicen.* The telson and uropods of the glaucothoe (G) and the first crab (C).

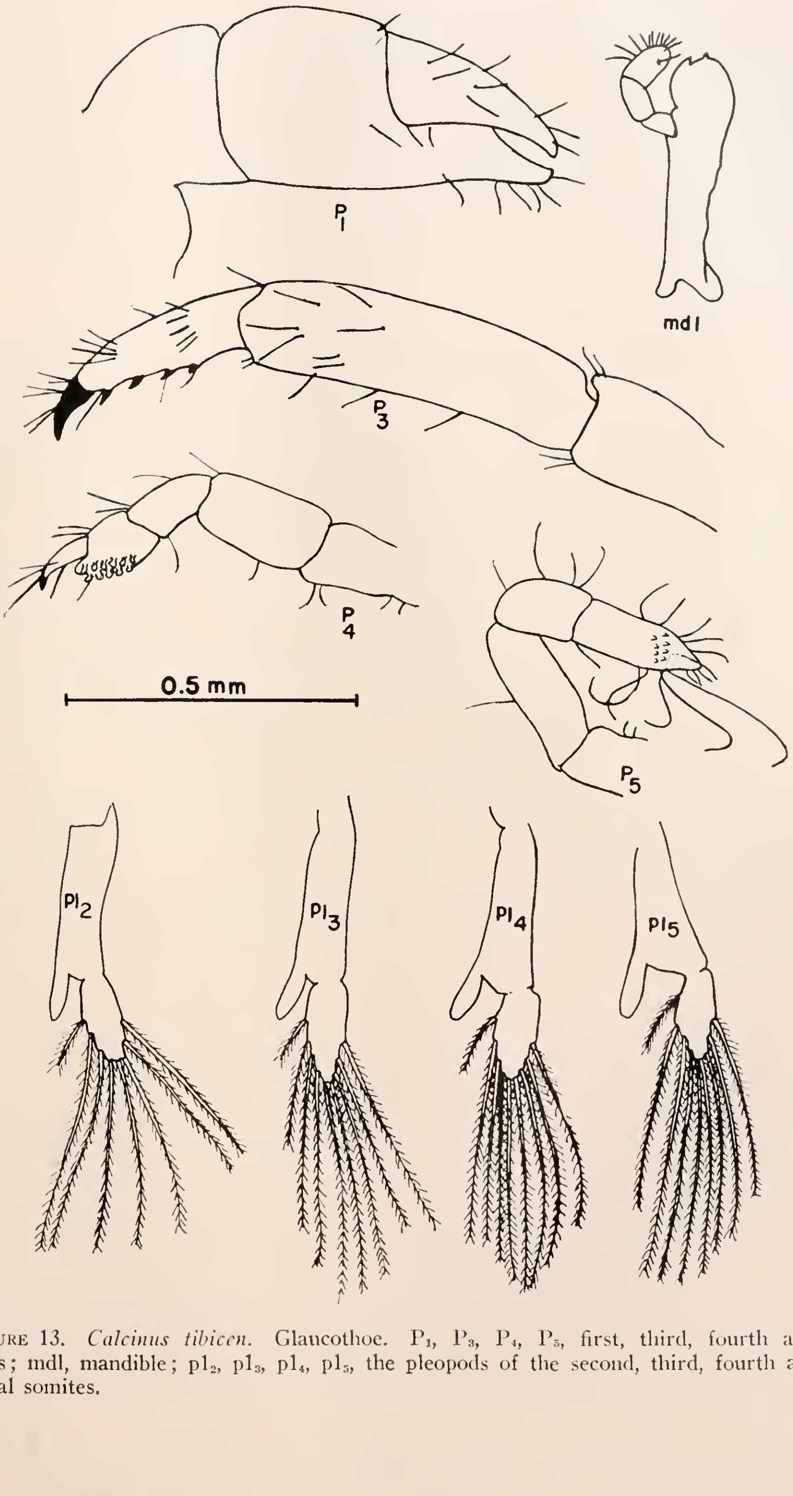
P1
mdl
P3
P4
0.5 mm
P5
Pl2
Pl3
Pl4
Pl5

FIGURE 13. *Calcinus tibicen.* Glaucothoe. P_1, P_3, P_4, P_5, first, third, fourth and fifth pereipods; mdl, mandible; pl_2, pl_3, pl_4, pl_5, the pleopods of the second, third, fourth and fifth abdominal somites.

up for it. Thus in one stage VIII specimen while the telson ratio of 2.6/1.4 indicated a slightly more elongate telson than in the final zoea of a series in which only six zoeal stages were found, the same animal had a telson armature count of 11 + 11 processes and the uropods bore 14–15 setae on the exopodites, 9–10 setae on the endopodites as in terminal stage VI. The antennal scale of a stage VIII may also resemble that of a terminal stage VI, having 16 plumose setae. In a series with eight zoeal stages the earlier stages at least as far back as stage III were somewhat less advanced than the corresponding stage of a shorter series. Table II summarizes changes of a few characters within one series.

Glaucothoe. Figs. 11, 12G, 13.
Size. TL 3.5–3.9 mm.; CL 1.4–1.6 mm.
Duration. This stage was attained 32–40 days after hatching for larvae which passed through only six zoeal stages, and after 39–42 days for larvae which passed through eight zoeal stages. Most specimens of the 1961 series died within 48 hours after attaining this stage. One glaucothoe lived for six days until killed accidentally. In the 1960 series one specimen attained the glaucothoe on the 37th day after hatching but lost one leg during transfer operations on the 47th day. Nevertheless the animal survived, regenerated the leg, and on the 57th day after hatching moulted to the first crab stage.

In *C. tibicen* the carapace has lost the postero-lateral spines and is now divided into sections. The rostrum is blunt but well developed. The pereiopods are free and functional, the chelipeds being subequal and the other legs symmetrical. The abdomen is not quite twice the length of the cephalothorax. The abdomen bears biramous pleopods on somites two to five, but only the exopodite of each carries setae.

The telson (Fig 12, G) is subrectangular, not indented as in the crab (Fig. 12, C), and may bear from 9–15 plumose setae terminally with two pairs laterally. Glaucothoes attained after zoea VI had 11 setae, one attained after VIII had 15 setae along the posterior margin. There may be a few pairs of smaller setae mediodorsally on the telson as well. The uropods are changed somewhat from the preceding zoeal stage and now bear corneous nodules along the posterior margin in addition to plumose setae. The exopodite may bear from 20–23 plumose setae plus a few very fine simple hairs. The endopodite may bear from 13 to 15 plumose setae as well as a few simple hairs.

The eyes, together with the eyestalks, are about two times longer than wide and are widest at the eyes.There are no ocular scales apparent. (Note: In the first crab stage the eyestalk is wider than the eye and there is a simple, acute scale at the base of each.)

The antennule (Fig. 4, G) is now distinctly biramous with three segments composing the internal or ventral ramus. The terminal segment may have up to nine small setae, the others two or three. The external or dorsal flagellum which arises from the same peduncular segment is composed of five articles. The most proximal is unarmed, the terminal segment bears a few fine setae. The intervening segments each bear a number of aesthetes.

The antenna (Fig. 5, G) reaches to the tips of the cheliped and has a scale at the base very different from the zoeal scale. In the endopodite there are two peduncular segments followed by about 10 flagellary segments, each of which bears a few short setae.

The mandible (Fig. 13, mdl) has a cuplike grinding surface and a three-segmented palp, the distal end of which bears 12–13 short setae.

The maxillule (Fig. 6, G) has lost the segmentation of the endopodite which now appears as a simple palp bearing a few setae. The basal endite bears a greatly increased number of stout spines and setae, about 17–21, distally and a pair proximally. The coxal endite also bears a greatly increased number of setae.

The maxilla (Fig. 7, G) bears a double row of setae on the proximal lobe of the coxal endite, about seven on the distal lobe. There is a large number of setae on the lobes of the basal endite but the endopodite no longer bears any setae. The scaphognathite is very well developed and has approximately 65 short plumose setae.

The first maxilliped (Fig. 8, G) is radically changed. The exopodite is short and broad, bearing seven plumose setae along the exterior margin. The endopodite is unsegmented and is devoid of setae. The basipodite appears bilobed and bears about 18 setae on the distal lobe and about six on the proximal lobe.

The second maxilliped (Fig. 9, G) is least changed of all the mouthparts. The exopodite is clearly unsegmented distally.

The third maxilliped (Fig. 10, G) is biramous and bears about six to eight plumose setae terminally on the exopodite. The ultimate and penultimate segments of the endopodite are heavily armed with setae, some of which bear setules. The antepenultimate segment has a row of distal setae while the proximal segment bears only three or four simple setae. The ischium has a row of fine tubercles or teeth along the medial margin.

The chelipeds are about equal in size, each with a few setae. The second and third pereiopods (Fig. 13) have the dactyli about half the length of the propodi. There are a few setae on the dactyl and four horny spines ventrally in addition to the corneous terminus. There are setae and a couple of short spines distally on the propodus. The fourth pereiopod is non-chelate and the propodus has a double row of about 15 corneous granules on the latero-ventral surface. The fifth pereiopod bears granules on both dactyl and propodus and very long setae are present on these segments.

The pleopods are biramous, the inner ramus being unarmed. The pleopod of the fifth somite is shorter than the preceding ones. The number of setae is eight on the pleopod of the second somite, nine on the others.

Discussion

The first stage larva attributed by Bourdillon-Casanova (1960) to *Calcinus ornatus* (Roux) bears very close resemblance to that described by Pike and Williamson (1960) from a laboratory hatching. There are minor differences in that the antennal scale has 11 plumose setae and the short seta of the antennal endopod is half as long as the others according to Bourdillon-Casanova, whereas Pike and Williamson reported nine plumose setae on the scale and the short seta as one fourth as long as the others. If these are in fact objective differences they may well be within range of variability for a single species as indicated in the present paper, but evaluation of such characters is hampered by the general lack of detailed descriptions of hermit crab larvae. The antennule of *C. ornatus* was not figured by Pike and Williamson, but Bourdillon-Casanova showed it terminating with three aesthetes and three setae in addition to a large plumose subterminal seta. In *C. tibicen* I was able to distinguish with certainty only one aesthete and four other processes which may have been aesthetes.

The combination of prominent postero-lateral carapace spines and the presence on the fifth abdominal somite of a single medio-dorsal spine and a pair of lateral spines is common to both species and may be unique to the genus, but Pike and Williamson also attributed an unidentified larva lacking the fifth abdominal medio-dorsal spine to *Calcinus*. In the first zoea of *C. ornatus* the medio-dorsal spine of the fifth abdominal somite is shorter than the pair of lateral spines, whereas in *C. tibicen* it is distinctly longer. The very small lateral spines or teeth on the second, third and fourth abdominal somites of *C. ornatus* were not distinguished in *C. tibicen.*

A character of questionable status is the endopodite of the maxillule which according to Pike and Williamson is two-segmented in *C. ornatus* and which in other Diogenidae never has more than two segments. In *C. tibicen* this endopodite appeared to have three segments in the specimens for which dissection of the appendage was successful. Hart (1937) reported three segments for the maxillulary endopodite of *Paguristes turgidus* and Orlamunder (1942) showed three segments for *Birgus latro. Coenobita clypeatus* also has been shown to have a three-segmented endopodite (Provenzano, in press). Both these latter species are members of the family Coenobitidae, presumably derived from the same line as the Diogenidae to which *Calcinus* belongs.

Comparison of later zoeal development of *C. tibicen* with that of other species of *Calcinus* is restricted to the work of Pike and Williamson. Those authors obtained their first stage larvae from a laboratory hatching and the subsequent stages from the plankton. They did not illustrate completely each stage including appendages but did describe certain essential features of each stage. Size of first stage larvae is similar for the two species, but the fifth and sixth zoeae of *C. tibicen* are slightly larger than the fifth zoea of *C. ornatus.* Other characters mentioned by those authors for later zoeal stages do not differ significantly from those of *C. tibicen,* with the exception that the postero-medio-dorsal spine on the sixth abdominal somite of *C. tibicen* is not certainly indicated in the illustrations of the later stages of *C. ornatus.* In both species the telson becomes more elongate during zoeal development.

The glaucothoes of the two species are very similar in gross appearance and this similarity may extend to details. Appendages are not illustrated or described in detail by Pike and Williamson but Bouvier (1922) for *Glaucothoe grimaldi* gave enlarged figures of the first, second, and fourth pereiopods, the antennule and antenna and the tail fan in addition to a figure of the whole animal. Between that form and the glaucothoe of *C. tibicen* there are a few minor differences which if later confirmed may prove to be significant at the specific level. Such differences include number of spines on the ventral margin of the ambulatory dactyl and relative lengths of some antennal segments, as well as certain setation. In all important respects, however, *Glaucothoe grimaldi* resembles the two glaucothoes certainly known for *Calcinus* and in fact, as Pike and Williamson have already indicated, Bouvier's form may belong to *C. ornatus.* Pike and Williamson stated that the glaucothoe of *C. ornatus* has the pleopods (each?) armed on the outer ramus with nine plumose setae, where in *C. tibicen* there are only eight on the pleopod of the second abdominal somite, nine on the others.

Insofar as the description and illustration of *C. ornatus* and *Glaucothoe grimaldi* indicate, the similarities between glaucothoes of several species may be much more significant than any differences. Until now glaucothoes of two species within one genus of Diogenidae have not been compared in detail. Rather the few descriptions available were for species of hermit crabs in different genera, and differences between most of these seemed to be quite distinctive. Whether those differences were generic in significance or merely specific could not be ascertained, especially since in *Paguristes,* the only diogenid genus for which larvae of more than one species were known, there seem to be notable differences between the larvae. Recently it was shown that zoeae and glaucothoes of two species of *Coenobita* are more similar to each other than to those of other genera of hermit crabs (Provenzano, in press). The present study also confirms that two species of a genus of diogenid hermits even as larvae bear more resemblance to each other than to members of other genera, a long accepted hypothesis for which there has been little direct evidence.

Summary and Conclusions

1. Larvae of *Calcinus tibicen* (Herbst) were reared in the laboratory from hatching to the post-larva on a diet of *Artemia* nauplii. Temperatures ranged from 26° to 30° C. Salinity in one experiment fluctuated between 31 ppt and 35 ppt and in the second experiment salinity was maintained between 35.5 ppt and 37.1 ppt. Under these conditions the zoeal phase of development was completed in 32 to 42 days. The number of zoeal stages in the laboratory development of this species is variable, the glaucothoe being attained after six, seven or eight zoeal stages.

2. Six zoeal stages and the glaucothoe have been described and illustrated. The only two species of *Calcinus* for which zoeae are certainly known share the apparently unique features of a pair of prominent submarginal postero-lateral spines on the carapace and a medio-dorsal and pair of lateral spines on the fifth abdominal somite. The species apparently differ in that *C. tibicen* has the postero-medio-dorsal spine of the fifth abdominal somite longer than the laterals, a postero-medio-dorsal spine is present on the sixth abdominal somite of zoea III and older larvae, the maxillulary endopodite is three-segmented, and there are no minute lateral spines on abdominal somites two to four.

3. Adequate comparisons of the zoeal appendages and glaucothoe characters of *C. tibicen* with those of other species are not possible until more detailed descriptions are available, but the information which has been published indicates close similarity of larval characters of species within a genus.

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ELECTRICAL INDUCTION OF SPAWNING IN TWO MARINE INVERTEBRATES (URECHIS UNICINCTUS, HERMAPHRODITIC MYTILUS EDULIS)

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The investigations of Iwata (1950) and Harvey (1952) have proved electrical stimulation to be a very effective means for inducing spawning of sexual cells in sea urchins. Recently, Kubota (1962) has reported the same effect in an insect (dragon-fly). Inspired by their success, the author tested the applicability of this method to various shore invertebrates, two successful cases of which will be briefly reported.

I. *Urechis unicinctus*

MATERIALS AND METHODS

By the ordinary practice, for obtaining reproductive cells of *Urechis*, either the body wall must be cut open or the eggs must be collected directly from the opening of the gonoduct by a fine pipette (Newby, 1932). When the electrical method is used, it is possible to obtain with ease any desired quantity of reproductive cells, and this can be applied repeatedly to the same individual, if necessary, since spawning lasts only during the stimulation. Moderate stimulation of the adults causes no harmful effects in the later development of the gametes.

The spawning season of *Urechis unicinctus* is said to be from October to March (Hiraiwa and Kawamura, 1936; Ohkawa, 1958; Sakiyama, 1958). At Kisarazu, Chiba Prefecture, where this study was performed, the rate of occurrence of sexually mature individuals remains fairly high even in April. Twenty animals (about 10 cm. in length in contracted state) were used.

One individual at a time is placed in a vessel (155 mm. in diameter, 33 mm. in depth) filled with sea water, and a pair of Ag-electrodes (1 mm. in diameter) are dipped vertically into the sea water 20 mm. apart across the body axis of the worm. The temperature and the specific gravity of the sea water are adjusted to 16.0–20.0° C. and 1.0209–1.0232, respectively, and alternating current (30 volts) is applied for various periods.

RESULTS

As soon as the stimulation is applied, the animal contracts quickly and in many cases a large swelling appears in the front of the body. About 4–20 seconds after stimulation, the animal begins to discharge reproductive cells forcibly through the openings of the gonoducts (Fig. 1). There is no conspicuous difference between females and males in their reaction to electrical stimulation.

On cessation of stimulation, the animal relaxes and after a while spawning also stops, a mass of sexual cells being deposited on the bottom of the vessel. A considerable amount of eggs or sperm can be obtained by a single discharge ranging from 5 to 35 seconds.

Over-stimulation lasting more than 30 seconds should be avoided, since this causes prolonged contraction or occasionally even death. The time required for recovery from the stimulation-induced contraction varied widely, from several minutes to several hours. Intermittent stimulations of very short duration seem to act excessively: after three stimulations of 6 seconds each, given 3–5 minutes apart, although all the animals discharged, 20% of them failed to recover from contraction.

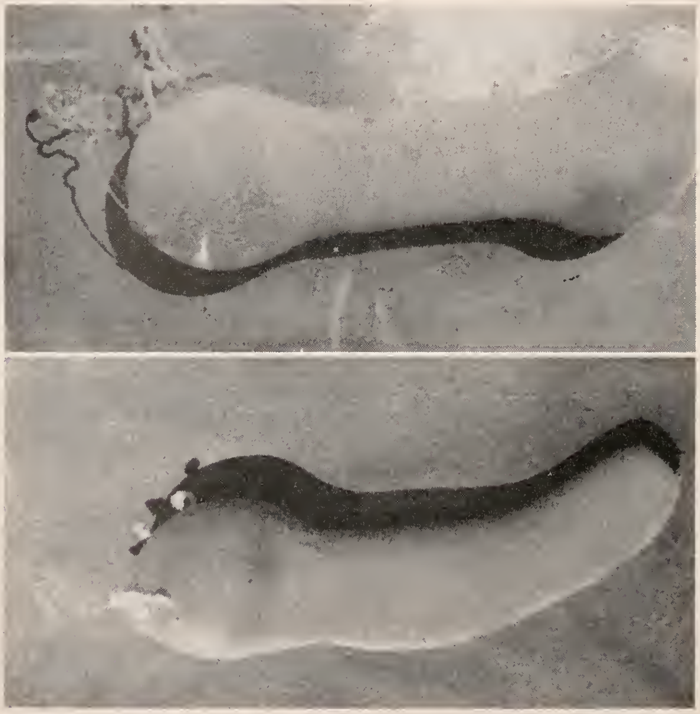

Figure 1. *Urechis unicinctus* spawning under electrical stimulation.

If due caution is paid to the duration of, and the intervals between, stimulations, one to two stimulations a day can be given for 6 consecutive days to animals fully charged with reproductive cells. In one case, animals which had been kept for 11 days in captivity and made to spawn nine times, still discharged eggs which developed on insemination, to normal trochophores.

II. Hermaphroditic *Mytilus edulis*

Materials and Methods

Mytilus edulis is normally dioecious, and hermaphroditic individuals can be found only very rarely. Among some 400 individuals examined by the author at

Kisarazu, Chiba Prefecture, four hermaphroditic mussels were met with (Fig. 2). They were detected by the colour of the gonads and the condition of the follicles, and further checked by smear preparations of the gametes.

With respect to the induction of spawning by electrical stimulation of *Mytilus*, Iwata's report is available (1949). In 1951, he further showed that discharge could be induced in excised parts of the mantle. Since *Mytilus* eggs are unfertilizable as taken from excised ovaries, requiring to be at least quasi-normally spawned, this method of Iwata's was used to obtain gametes from such hermaphroditic individuals.

Pieces of the mantle about 5 mm.^{2}, containing either ovary or testis, were cut out of a hermaphroditic individual. Each piece was stimulated separately by 40 volts a. c. for 15 seconds with a pair of Ag-electrodes 1 mm. in diameter, placed 20 mm. apart in a vessel of 72 mm. diameter filled with sea water to a depth of 5 mm. After a latent period of about 30 minutes (19° C.), eggs and sperm were spawned; these were used for cross-fertilization in various combinations. In no case did eggs to which sperm was not added show any development, indicating that they were not contaminated with sperm during spawning.

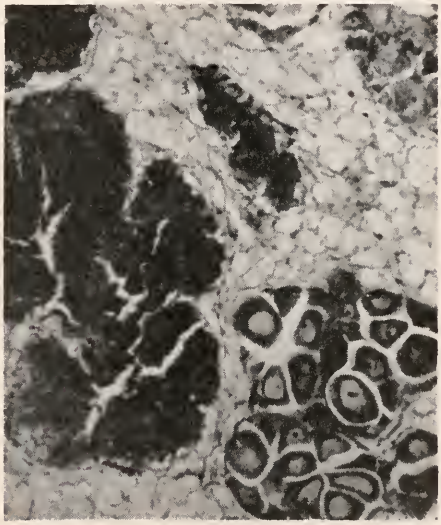

FIGURE 2. Hermaphroditic gonad.

RESULTS

Self- and cross-fertility

Separately obtained gametes were mixed in various combinations and the percentages of resulting fertilization were counted. The results are summarized in Table I. Later development of such zygotes was followed; in all four hermaphroditic individuals, self-fertilization zygotes were found to develop at the normal pace, and the embryos showed no difference whatsoever from out-bred controls.

Gametes were sometimes obtained from an ovary and a testis situated in the mantels of opposite valves and in other cases they occurred together on the same side;

TABLE I

Fertilizability of gametes from hermaphroditic mussels

Combination	Fertilization percentage
Herm. egg × herm. sperm	92.8%
Normal egg × herm. sperm	94.0%
Herm. egg × normal sperm	93.2%
Normal egg × normal sperm	96.0%

in both cases the results were identical. In one mussel, a piece of the mantle containing gonads of both sexes was stimulated and normal larvae were obtained without further insemination.

These facts lead to the conclusion that the gametes of hermaphroditic *Mytilus edulis* are capable of self-fertilization which produces perfectly normally developing larvae.

The author wishes to acknowledge his indebtedness to Prof. K. Dan of Tokyo Metropolitan University for his encouragement and advice during this work. This research was carried out when the author was at the Kisarazu Fisheries Laboratory of Tokyo University of Fisheries. Thanks are due to Prof. M. Katada, director of the Laboratory, and Mr. I. Shimizu of Funabashi Senior High School, who cooperated with the author in this work.

SUMMARY

1. Electrical stimulation of spawning was tried on *Urechis unicinctus* and hermaphroditic *Mytilus edulis*.

2. *Urechis* reacts immediately, and if over-stimulation is guarded against, samples can be repeatedly obtained from the same individual, which would be very advantageous for some kinds of experiments.

3. Excised pieces of hermaphroditic *Mytilus* were stimulated electrically to spawn; the gametes so obtained showed perfect self-fertility.

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SOME OBSERVATIONS ON THE GENERAL BIOLOGY OF THE LAND CRAB, CARDISOMA GUANHUMI (LATREILLE), IN SOUTH FLORIDA [1]

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Land crabs of the genus *Cardisoma*, Family Gecarcinidae, are an important element of the fauna of many tropical coastal and estuarine areas. The genus is circum-equatorial, with different species on the east and west coasts of each continent. *Cardisoma guanhumi* was described by Rathbun (1918). Its range includes the east coast of America, from Florida to Brazil, and the Caribbean Islands.

Despite the large numbers in which *Cardisoma* occurs in or near many densely populated areas, and its spectacular colors, migrations, and swarming, it has been largely neglected by zoologists. Brief descriptions of its ecology and behavior were given by Pearse (1915) and by de Oliviera (1946). Pearse (1934) found its blood to be hypo-osmotic to normal sea water. The eggs and first zoeal larva of the West African species, *C. armatum*, were described by Cannon (1923). Taxonomic descriptions and reports of occurrence are more frequent and sometimes include brief notes on habits and ecology.

Some aspects of the physiology of blood regulation of *C. guanhumi* have been studied in this laboratory and will be published elsewhere. The following observations were made during the collection and maintenance of a stock of animals in the laboratory. Since Florida is at the northern limit of the animal's range, these observations may not apply to the species as a whole.

General Description

C. guanhumi was described in detail by Rathbun (1918). As an adult it somewhat resembles *Uca* in general shape; its eyes are widespread, large and on fairly long stalks, and one cheliped of adult males is enlarged. *Cardisoma*, however, is much larger than *Uca*. Adult males weighing 500 grams, with carapace widths of 10–11 cm., are not uncommon. Crabs weighing 4–5 grams are also common, although more difficult to capture.

Habitats

Geographic range

C. guanhumi has been seen by the author, or reported to him by competent observers, from Vero Beach on the central east coast of Florida around the tip of the peninsula, in the Florida Keys, and along the Gulf coast as far north as Tampa. It was reported from Louisiana (Behre, 1949), and has been seen by the author at Rockport, Texas. It has not been seen more than eight kilometers inland.

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Salinity range and tolerance of desiccation

In south Florida the salinity range of the surface water nearest to *Cardisoma* burrows varies from fresh water (Cl = 0.4 m*M*/L.) to slightly concentrated sea water (Cl about 600 m*M*/L.). This range, and its effect on blood composition, will be discussed in greater detail in later papers. Crabs have been reported on the ocean bottom several hundred meters offshore in the Florida Keys (L. Greenfield, personal communication). During extended droughts crabs living inland feed on plants on the bottoms of fresh-water drainage ditches and canals. Individual *Cardisoma* have been kept completely immersed in a running sea water aquarium for six months, but tolerance of extended immersion in large volumes of fresh water has not been tested.

When permitted, *Cardisoma* spends most of its time out of water. In a box containing a shallow pan of water, *C. guanhumi* will approach the pan, dip its small chela in the water, then touch the moistened chela to the borders of the buccal cavity and the maxillipeds. Over the salinity range from tap water to 150% sea water, the crab then enters the pan, lowers its body until the ventral margin of the carapace is immersed, and fills its gill chambers. It then leaves the pan, and within a few minutes drains the gill chambers by elevating the front of the carapace. The latter is firmly attached posteriorly, but it is free to lift slightly at the anterior end, an action which allows most of the water to drain from the gill chambers. A recording device measuring the frequency and duration of such immersions by a crab given access to 0.5% sea water showed that most occurred at about the time of sunrise and sunset. The average time of immersion was about two hours per day under the conditions of the test.

Cardisoma can live for many days in moist air, but only for about two days under severe desiccation. A group of eight crabs contained 60–70% water (on original weight basis) and lost 10–15% of their original weight at the time of death by desiccation.

Colonies

In south Florida *Cardisoma* lives in two major types of colonies. In one of these, the burrows are located in flat ground not immediately adjacent to free surface water. These may be either among mangroves, in open fields of tall grass, or in open hardwood groves. Among mangroves the burrows are usually on ridges of comparatively high ground. The burrows extend to ground water, which may be from one-third to two meters below the surface. The upper part of the burrow is generally vertical or nearly so. In local colonies of this type, burrow density may average one per square meter over areas of several hectares. These colonies are generally within half a kilometer of some kind of free surface water. *Cardisoma* burrows have been found in many types of soil, but the largest colonies of this type are found in Perrine marl (described by Gallatin, 1958).

In the other type of colony, the burrows are located in the banks of drainage ditches or canals, near the edges of fresh-water streams or ponds, or in hard soil near salt water. The burrow can be either in the vertical face of the bank extending horizontally into it, or on level ground immediately adjacent to the bank, when the burrow tends toward the vertical. Burrow density frequently exceeds one per square meter in this type of colony. Quite often the burrows are as close together as they can be without inter-connecting or collapsing.

Both habitats are shared with other animals. Raccoons (*Procyon lotor*) are common in the first type, and may be an important predator of *Cardisoma*. Gray squirrels (*Citellus carolinensis*), rabbits (*Sylvilagus* sp.), and rats (*Rattus* sp.) also occur there. The blue crab, *Callinectes sapidus,* is occasionally seen in the ditches, as are prawns of the genera *Palaemontes* and *Macrobrachium,* and the crayfish, *Procambarus allenii.* On the seaward border of its range *Cardisoma* is sympatric with several species of *Uca*, and its habitat may border that of *Ocypode albicans.*

LIFE CYCLE

Mating, ovulation, and fertilization

The time, place, and manner of copulation are unknown. The sperm are carried by the female in a spermatheca, and fertilization is internal. In seven animals the egg mass weighed 11.9% (± 2.2) of the body weight. In five females the egg cluster, just prior to spawning, contained 19,000 to 20,000 fertilized eggs/gm. of egg cluster. A female weighing 160 grams would thus release about 370,000 eggs at each spawning. The diameters of ten eggs ranged from 430 to 440 mu.

The spawning period extends from June or early July to December, with a peak in October and November. Spawning occurs in waves which appear to have a lunar or twice monthly rhythm. These are described below, under migrations. A more detailed report of the female reproductive cycle is in preparation.

Adult females generally change color from blue to yellowish-white, at about the time of the first ovulation of the season, and the lighter color generally persists through the season. The females which have spawned are thus effectively tagged. Ovaries of such crabs, captured between the July and August, 1961, spawning periods, were again beginning to mature, suggesting that individual females may spawn more than once during a single season.

Cyclic changes in the male reproductive system are either absent or less pronounced than the female.

Fertilized eggs were carried on abdominal appendages for ten days by one female crab which ovulated in captivity. When first deposited, the egg mass is black, compact, and shiny. As development proceeds, it becomes loose and ragged, and light brown in color. The embryos, by then, have reached the pre-zoea or zoea stage. At this time the females generally start migrating to salt water to shed their young, seldom traveling more than five to six kilometers. This distance must be accomplished in one night, or at most two, because if ovigerous females at this stage are captured and held overnight, the egg mass usually disintegrates. The females seek cover near the water's edge and periodically enter the water and release the eggs or larvae by rapid fanning movements of the abdomen.

Attempts to rear the larvae have been unsuccessful, and the foods suggested by Costlow and Bookhout (1960) do not sustain development. The larvae may ingest yeast cells or other microorganisms and produce fecal pellets or strings, but only a few have survived the first molt. The survivors spent 5–7 days in the first zoeal stage. Cannon's drawings (1923) of the eggs and first zoeal stage of the West African species, *C. armatum,* closely resemble the corresponding stages of *C. guanhumi.*

Attempts to hatch *C. guanhumi* eggs in drainage ditch water brought to the laboratory were unsuccessful. Only a few eggs hatched, and these were pre-zoeal which soon died. When tested in increments of 10% sea water, both hatching and survival improved with increasing salinity. All eggs hatched in 40% sea water (diluted with drainage ditch water), but the larvae lived only a few days. The percentage of eggs which hatched declined progressively in salinities over 50% sea water, but the larvae survived longest (7–10 days) in 80–90% sea water. This was also true of larvae hatched in 40% sea water and transferred to 90% sea water. The main spawning period occurs at the height of the rainy season, when littoral salinity may be decreased by run-off. The ability of the larvae to hatch and survive in brackish water may thus have adaptive value.

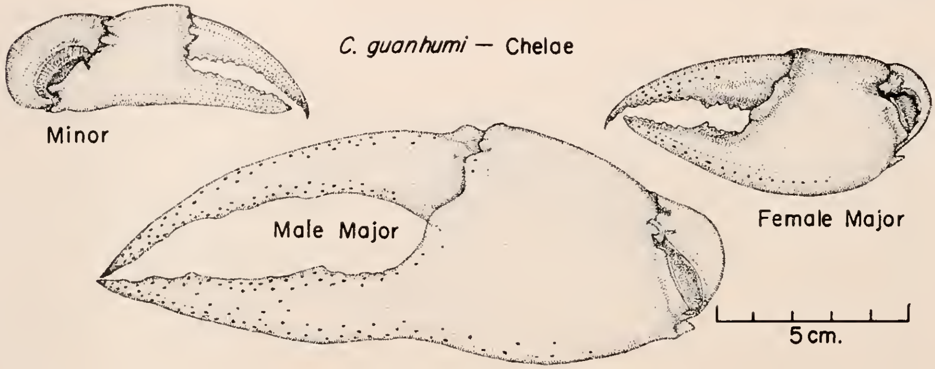


FIGURE 1. Shapes of major and minor chelae of *C. guanhumi.* Distribution of types of major chelae with growth stage is explained in the text.

The duration of the larval period is unknown, but it may be several months. In May, 1961, a group of very small crabs (<5 grams) appeared on an exposed drainage ditch bank a kilometer or so inland. Ovigerous females had not been seen since December, 1960, five months previously. It would appear that it had taken these specimens at least five months to develop from zoea larvae to five-gram crabs, or to have colonized the area from some other center.

Post-larval life

In its growth from small crab to adult, *Cardisoma* undergoes a series of morphological changes. Post-larval life can be divided into three stages, juvenile, transitional, and adult on the basis of the following characteristics: (1) shape and size of the major chela; (2) shape of the carapace.

Juvenile: In the smallest crabs captured (<5 grams), the sexes can be differentiated only by retracting the abdomen, at this stage resembling that of the adult male, and identifying the female genital pores on the under surface of the third segment of the cephalo-thorax, or the male copulatory organs on the first abdominal segment. The chelipeds are unequal in both males and females, with the major one resembling that of the adult male (see Fig. 1). The minor chelipeds are similar in the two sexes, but as growth proceeds the major ones come to differ in both size and shape.

As is shown in Figure 2, the shape of the carapace changes with growth. The ratio, carapace width/carapace length is fairly constant throughout life (C.W./C.L. = 1.222 ± 0.022 for 73 male and female crabs weighing from 3 to 500 grams), but the junction between dorsal and lateral surfaces becomes rounded and the gill chamber covering bulges out farther to the side. This change in shape is further illustrated in Figure 3, in which the ratio of carapace width to orbital width (the distance between the tips of the innermost of the two epibranchial teeth projecting from the lateral borders of the orbits) is plotted against the logarithm of weight. Mean values of this ratio for both male and female crabs increase linearly from 1.12 at five grams to about 1.18 at forty grams.

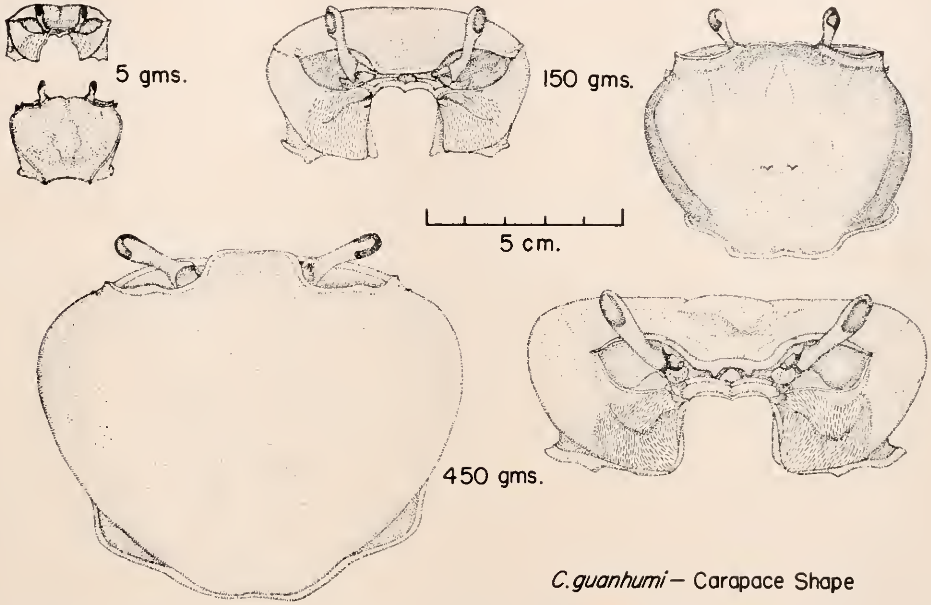


Figure 2. Differences in carapace shape of male *C. guanhumi* of different live weights. The carapace is often asymmetrical, bulging out farther on the side toward the major cheliped.

At succeeding molts the female abdomen widens, becoming first triangular and then semi-elliptical, and the ratio C.W./O.W. increases. By the time the body weight reaches 40 grams the sexes can be distinguished by abdominal shape. The chelipeds of both sexes still resemble those of the adult female. The junction of the dorsal and lateral carapace surfaces remains angular, and is defined by a ridged suture. Attainment of 40 grams live weight marks the end of the juvenile period. Color also changes with growth; these changes are described below.

Transitional: The beginning of the transitional period, at about 40 grams live weight, is marked by the beginning of major sexual dimorphism, and by an abrupt increase in the width of the carapace compared to orbital width (Figs. 2 and 3). Below 50 grams the mean values of the ratio C.W./O.W. are similar for males and females. Between 50 and 200 grams the mean values of C.W./O.W. are consistently higher for males, although standard deviations overlap. Above 200 grams, values for both sexes are erratic and displaced above the lines established between 50 and 200 grams.

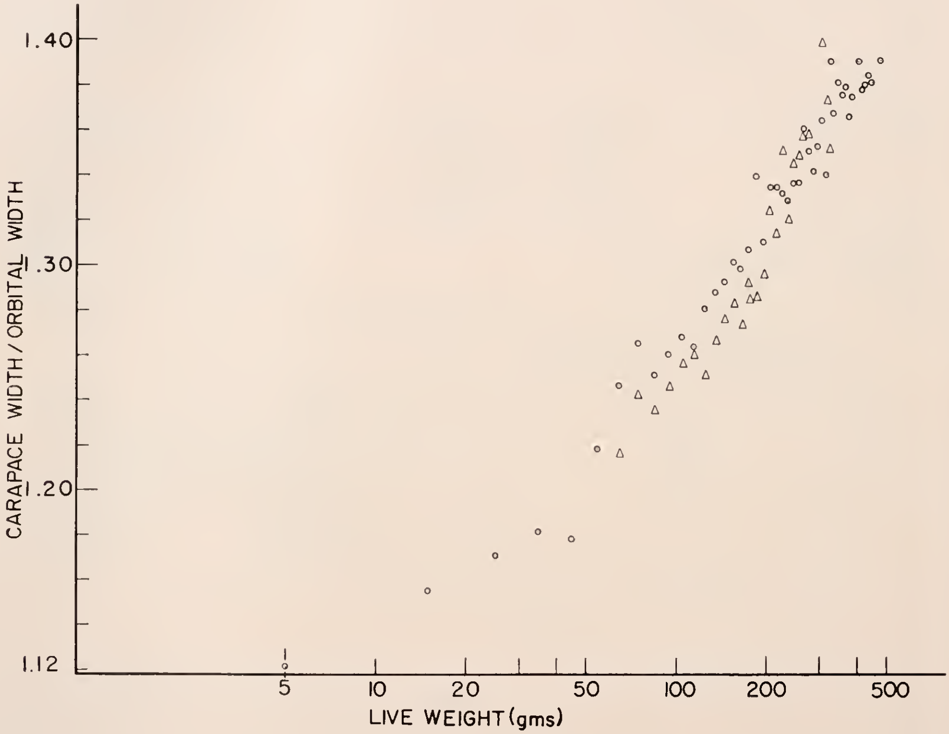


FIGURE 3. The ratio, carapace width/orbital width, plotted against log live weight. Circles represent mean values for from 7 to 22 males in 10-gm. weight increments. Triangles represent similar groups of females. Standard deviations averaged ± 0.022 for all groups. Below 50 gm. both sexes are represented by circles.

Other morphological changes which occur in the transitional period are a gradual rounding of the junction of the dorsal and lateral carapace surfaces, the loss or diminution of the second epibranchial tooth (this occurs earlier and more consistently in males), and, in the male, the enlargement and change of shape of the major cheliped. Transitional males sometimes have identical chelipeds, or, if they are unequal in size, the major one may have the same shape as the minor. The range of shapes is shown in Figure 1. The major cheliped of the male is generally recognizably different from that of the female when body weight reaches 120 grams, but great enlargement of it is rare in crabs weighing less than 200 grams.

Adult: There is no sharp morphological change from transitional to adult. The major change in outward appearance is in color, which is described in a later section. The morphological changes described above are gradual, and, from the limited observations made, do not occur in any particular order. Females can attain sexual maturity and spawn at any weight over 40 grams. The size at which males achieve sexual maturity is unknown.

Handedness

The major chela can occur on either the right or left side of the animal. Of 562 crabs examined, 302, or 53.7%, were right-handed; 244, or 43.4% were left-handed, and 16, or 2.1% had equal-sized chela.

Molting

The difficulty of ascertaining the molting habits of *Cardisoma* has been mentioned by Schmidt (1934). Although empty carapaces are often found in the crab colonies, no intact cast shell has been seen. One juvenile crab has been forced to molt in captivity by removal of four pereiopods followed by continuous feeding. Destalked crabs have not lived more than three weeks after operation, and none of these has shown any outward sign of approaching molt before death. One crab with an uncalcified carapace, but with hardened mandibles and legs, was captured as it left its burrow and entered a salt water pond. Many "new-looking" crabs, with bright colors, unscratched carapaces, and no missing appendages, were seen when the crabs first became active in the spring of 1961. Molting probably occurs in the depths of the burrow.

TABLE I

Distribution of color phases in different weight and sex groups of Cardisoma guanhumi
(Total number of crabs examined: male, 454, female, 309)

Color Phase
(Number of cases)

Weight (gm.)	Juvenile		Transitional		Adult	
	Male	Female	Male	Female	Male	Female
0–39.9	27	20	0	0	0	0
40.0–79.9	0	0	0	12	4	5
80.0–129.9	0	0	7	8	28	38
130.0–189.9	0	0	2	23	52	125
Over 190.0	0	0	0	0	325	78

Coloration

In juvenile *C. guanhumi* the dorsal surface of the carapace is tan or brown with scattered small purple spots over the visceral mass. The orbits and eyestalks are purple, and the sides of the carapace and the tips of the chelae are white. The walking legs and the proximal segments of the chelipeds can be any combination of light blue, pale orange, and tan. Transitionals are similar to juveniles, but the colors are more intense. The orbital purple band extends laterally and posteriorly over the gill chambers, and down over the sides of the carapace. The middle segments of the pereiopods are purple. In both juveniles and transitionals, the ischium of the third maxilliped is white and all the more distal segments are bright orange red. An orange band frequently extends around the lower edge of the carapace, especially at its posterior margin, and over the proximal ends of the meri of the walking legs.

Adults are generally blue, but may be mottled blue and white or blue and dull yellow, or any shade between completely white and completely dull yellow. The chelae are white or yellowish-white. The maxillipeds are blue, white, or yellow, matching the rest of the crab.

Table II

Sex and color distribution of Cardisoma guanhumi collected near the bay shore during a migration, August 30, 1961

Color	Male			Female		
	No. of crabs	% of males	% of total	No. of crabs	% of females	% of total
Blue	121	94.5	39.4	121	67.7	39.4
White	1	0.8	0.4	32	17.9	10.4
Purple	5	3.9	1.6	22	12.3	7.2
Brown	1	0.8	0.4	4	2.2	1.3
	128		41.8	179		58.3

The distribution of color stages of *C. guanhumi* with weight and sex is given in Table I, and the distribution of color in a group of migrating crabs is given in Table II. Most crabs develop the adult color pattern by the time they weigh 80–90 grams, but some do not until they reach 180 grams. A higher proportion of these are female. Females generally have the adult color scheme before reaching sexual maturity.

These color patterns are achieved by different combinations of the effects of pigments embedded in the shell and of epidermal chromatophores. Briefly, juveniles use both means of coloration, shell pigments are more important in transitionals, and adult coloration is exclusively due to pigments in epidermal chromatophores.

The pigment of the shell occurs just below the epicuticle. In histological sections this layer stains differently than the rest of the shell. Two types of shell pigment have been observed in *C. guanhumi:* a continuous brown, orange, or yellow layer most prominent in juveniles, and a stratum of discrete round or irregularly shaped spots of purple or violet pigment, which may or may not be superimposed on the brown continuous layer. The latter is most evident on the carapace of transitionals. Where the two types occur together, as on the dorsal surface of the carapace of juveniles and transitionals, the purple spots extend farther into the shell than the brown layer does. In the orbits and on the eyestalks of juvenile and transitional crabs the purple spots occur alone, and merge into a continuous layer. These pigments are absent in adults. Since a series of shells has been found containing gradually decreasing amounts of pigment, more than one molt may be required for their removal.

Monochromatic red and black chromatophores can be seen in the pereiopods and gill chamber linings of juvenile *C. guanhumi*. These are embedded in a diffuse layer of white pigment that may or may not be in chromatophores; it occurs in fine filaments, but has not been seen to contract. In intact crabs in daylight both colors of chromatophores are contracted. The red chromatophores are much smaller than the blacks, and about six times as numerous. In darkness, or following eyestalk removal, both types expand, but even when fully expanded the blacks do not overlap.

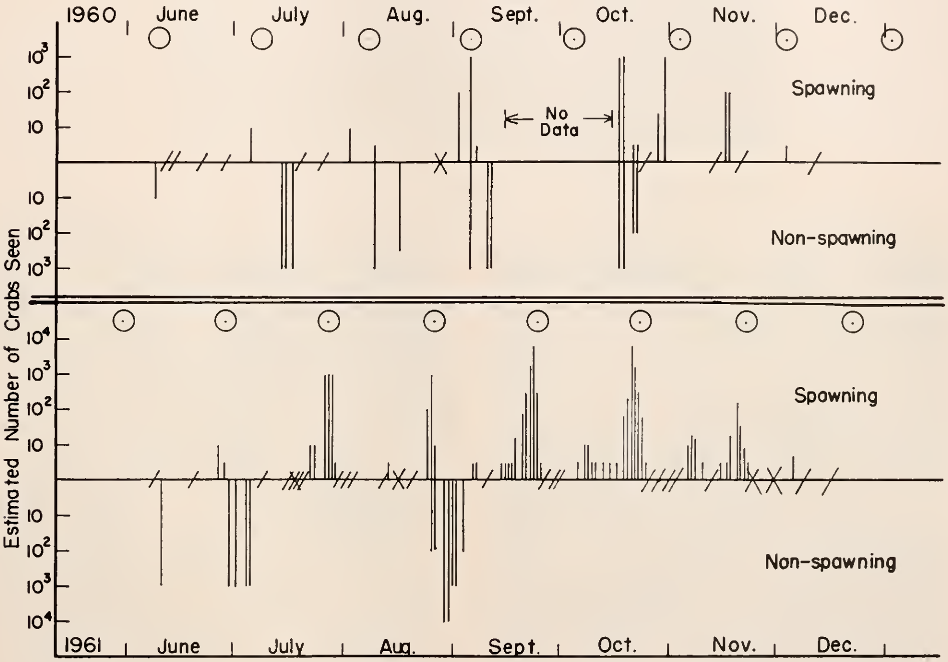
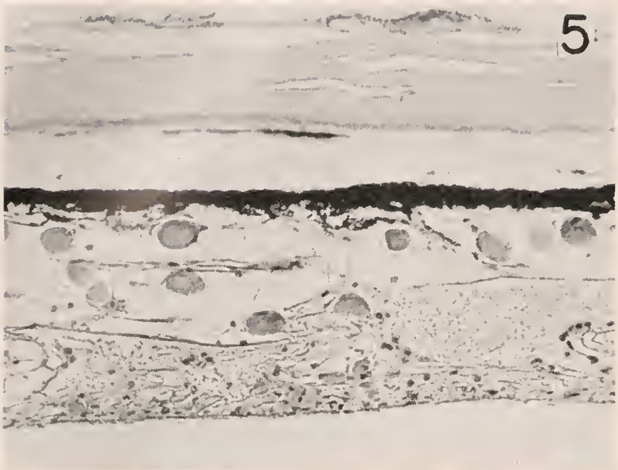


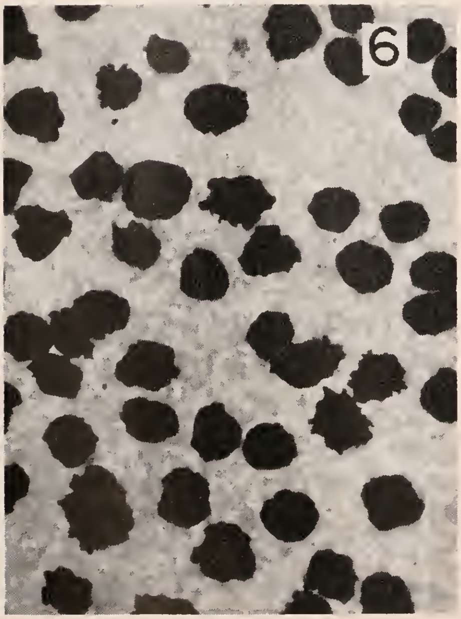
FIGURE 4. The frequency and intensity of migrations of *C. guanhumi* in the periods from May to December, 1960 and 1961. Diagonal marks indicate dates when no migrating crabs were seen. X indicates dates when no crabs were seen. Circles indicate dates of full moon. In 1960 numbers greater than 10^3 were not estimated.

In transitionals the chromatophores are obscured by the shell pigments described above.

Adult *C. guanhumi* are all deep blue when they first become active at the end of the dry season in late May. This color is due to a dense layer of expanded black chromatophores closely applied to the inner surface of the membranous layer of the shell. This layer can be observed, either in histological section as in Figure 5, or by removing and opening a walking leg and viewing the exposed epidermis perpendicularly from its inner surface by transmitted light. In deep blue crabs only expanded black chromatophores can be seen, embedded in a filamentous white pigment similar to that seen in juveniles.

Variations in adult color appear during the summer and fall. At some time





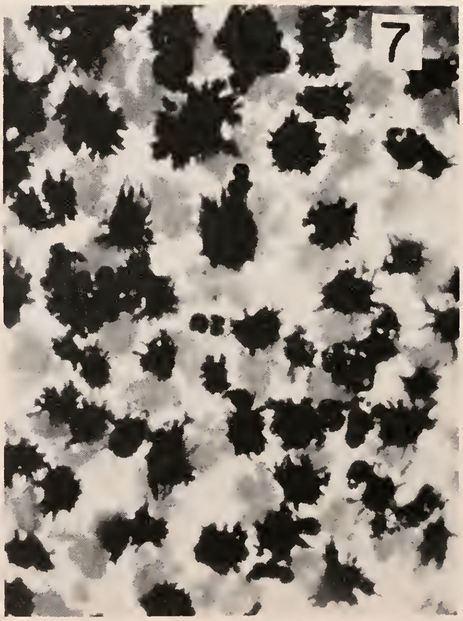


FIGURE 5. Section of the epidermal lining of the gill chamber (× 225) showing a dense layer of expanded black chromatophores immediately under the membranous layer of the shell. The calcified layers of the shell were removed to facilitate sectioning. The separation between the membranous layer and the epidermis is an artifact of fixation. The large cells embedded in the epidermis are not chromatophores. The outward color of the crab was blue.

FIGURE 6. The epidermis of a walking leg viewed from its inner surface by transmitted light. (× 140). The black chromatophores are withdrawn from the membranous layer and contracted. A layer of expanded yellow chromatophores is located closest to the shell. The outward color was yellow.

FIGURE 7. Same as Figure 6 (× 140), but with both black and yellow chromatophores nearly contracted. The outward color was white.

between ovulation and spawning, most females turn from the normal deep blue to either white or yellow. Intergrades between all three colors have been seen in ovigerous females. When examined as described above, the epidermis of these crabs displays both yellow and black chromatophores in different stages of expansion. In some preparations faint white contracted chromatophores can be seen on the inward side of the blacks when the preparation is viewed with reflected light. The yellow chromatophores resemble the blacks in number and size, but are separate from them and located closer to the inner surface of the shell. Unless both types are fully expanded the yellow chromatophores can be seen through or between the blacks. Various combinations of expansion of the two types are shown in Figures 6 and 7. They account for the color stages between blue and yellow. In females the whole surface of the crab is usually more or less the same color. While deep blue or bright yellow crabs do not undergo rapid color changes, even when destalked, intermediate color stages can change from blue-gray to yellowish white in an hour or so. The percentage of white females in the population increases after each spawning period.

Males also change from blue to white or yellow, but only in the fall, and then not as frequently as females do. While completely white or yellow males have been seen, blue males mottled with patches of white or dull yellow, or with one or more white legs, are more common.

In a small fraction (less than 6%) of the adult population, the dorsal surface of the carapace and the distal segments of the legs are dark brown or orange-brown. The sides of the carapace and the upper segments of the walking legs are lighter brown, and frequently have a faint lavender tone. The brown color is in the shell and the epidermal pigments do not show through, hence females of this group do not change color when they spawn. The eyes are always brown or yellow in contrast to the normal black. The migratory pigments seem to be withdrawn or absent. The chelipeds of adult males of this color pattern are often equal in size and shape, in contrast to the normal asymmetry. The outer margins of all but the dactyls of the walking legs have many conspicuous clusters of long, fine, black bristles. Juveniles and transitionals of this group are bright orange-red all over, in contrast to the normal purple-brown-orange color scheme. At maturation the third maxillipeds of this group change from orange to brown. Ovigerous females of this aberrant group have been found during the normal spawning periods, but the following slight differences from normal spawning have been observed. In the first spawning period of 1961 (in late June) over half of the females captured during the first two days were of this type, although they constitute only about 2% of the female population. In the third spawning period (in late August) several ovigerous crabs of this type were captured several days after the normal spawning migration ended. None were seen during the main spawning periods in September and October of 1961.

HABITS

Migration and swarming

The observation area extends roughly thirteen kilometers along Biscayne Bay and from three to five kilometers inland. It has a dense crab population, and contains a variety of habitats.

Exploratory daylight trips were made to locate areas of the most dense population and to capture crabs for use in the laboratory. It was soon found that crabs in colonies dispersed through fields and woods could be captured only by trapping, which was non-selective and slow. Subsequent effort was concentrated on the canal bank colonies, which are usually bordered by some sort of road. Crabs can be captured easily at night in these roads because automobile headlights or a powerful flashlight seem to dazzle or confuse them. On crab-collecting trips, the number of crabs moving, the types of activity, and the sex, size, and color of the crabs captured were recorded. Migrations were detected by observing the numbers of crabs crossing the roads near the colonies or accumulating in large numbers in different parts of the area. A fixed route was followed, and observation and collecting trips were generally made within three hours after sunset. Individuals were not tagged, so this method can only detect mass migrations. As a pattern of activity began to emerge, specific observation trips were made to determine types of activity, periodicity, and the number of crabs participating.

In south Florida *C. guanhumi* is more active in the wet season (May to December) than in the dry season. This pattern can be modified by unseasonal rains or droughts, but during February, March, and April the crab tends to stay in its burrow and feeding is greatly reduced or stopped. At both ends of the dry season more crabs can be seen feeding under water than on land. During this period of decreased activity, crabs often can be seen just inside the burrow entrance. The burrow entrances are generally kept open and free of debris, or closed a few centimeters from the surface with mud brought up from underground. The number of closed burrows increases in the dry season, but they are never all closed or all open.

During the rainy season *Cardisoma* displays several types of activity. Crabs in the canal bank colonies are generally nocturnal, but in one wooded field colony this is reversed: crabs are active during the day and inactive at night. At the peak of the largest mass migrations crabs also keep moving during the day, but in both cases of daylight activity they seek shade at mid-day.

Crabs may remain in the burrow, feed within a few feet of the burrow, join other crabs in swarming within 50 feet or so of the canal bank, or join other crabs in mass migrations. The intensity and duration in time of the major episodes of swarming and migration in 1960 and 1961 are shown in Figure 4. These periods can be divided into two categories: spawning migrations involving only ovigerous females, and mass migrations or swarming involving a cross-section of the whole adult population.

The spawning season extends from late June to early December, but is not continuous. Spawning occurs in sharp peaks near the time of the full moon. The number of ovigerous females recorded in each period increases to a maximum in September and October, then declines. During each period ovigerous females appear simultaneously over the whole inland portion of the study area four to six days before the full moon. Concentration may vary considerably from one place to another. During the following nights the number of ovigerous females moving increases, generally reaching a peak between one night before the full moon and one night after. By the second night of the period large numbers begin to accumulate at the bay shore. By the night of the full moon many of these have spawned and a few are beginning to move back inland.

The spawning period ends abruptly. Usually only a few ovigerous females could be found in the study area 48 hours after the peak of a spawning period. The gradual termination of the October, 1961, period may have been due to a sudden

20° F. drop in temperature which occurred two days before the full moon. Recently spawned females can be seen crossing roads parallel to the bay shore for several nights after the end of the spawning period.

Semi-lunar spawning periods also occur, but these are not as consistent, as intense, or of as long duration as the lunar peaks. In 1960 they were recorded midway in the lunar cycle in October and November. In 1961 they occurred from August through December, the end of the observation period. In October, 1961, a few (<5) ovigerous females were found on each of four census trips between the semi-lunar and lunar spawning periods. On the first three nights of the October lunar spawning period the numbers of ovigerous females seen were 83, 296, and an estimated 8000, respectively.

In the canal bank colonies activities of crabs range from complete inactivity to mass migration. Nights on which a standard trip through the study area revealed 100 or more non-ovigerous crabs crossing roads 50 yards or more from the nearest colony, or congregating in areas of low burrow density, have been arbitrarily called non-spawning migrations, and are recorded as such in Figure 4.

Four non-spawning migrations occurred in 1960 and two in 1961. All of these started at the time of, or shortly after, a spawning migration. The two large non-spawning migrations in 1961 were most closely observed and followed a common pattern.

While the spawning migration is in progress, transitional and adult males and non-ovigerous females congregate at the inland ends of some of the drainage ditches and canals leading back from the bay. At about the time that spawning ends, they leave the canal heads and radiate out, many of them going inland. Two or three days later large numbers appear along the bay edge. They are active during the day, and at low tide they can be seen crossing the exposed mud flats and entering the water. They have also been seen in the upper branchs of mangrove trees at this time, although they do not normally climb trees. Within a day or two the swarm at the bay edge disappears, and for several nights following large numbers of crabs can be seen crossing inland roads running parallel to the bay. A few crabs could always be seen along the canals, and one wooded field colony has always had a crab in every burrow inspected while the migrations were in progress.

The first part of this sequence, congregation at certain canal and ditch heads and swarming in adjacent roads and fields, occurred during all of the spawning migrations in 1961, but only developed into mass non-spawning migrations after the July and August spawning migrations.

The sex and color distribution in a sample (307 crabs) taken near the bay just after the peak of the August, 1961, migration is given in Table II. Some recently spawned (white) females were found, along with transitionals (purple) of both sexes but the largest fraction consisted of equal numbers of adult (blue) males and females.

Spawning migrations differ from non-spawning in occurring more regularly and uniformly over the whole study area, in being of shorter duration, and in beginning and ending more abruptly.

Discussion

Physiological, anatomical, and behavioral adaptations of crustaceans to terrestrial life have been reviewed by Edney (1960). "Terrestrialness" among decapods ranges from generally intertidal genera like *Sesarma, Pachygrapsus,* and *Uca* to forms such as *Birgus* and *Gecarcinus* which enter water only to spawn, and perhaps to molt. *C. guanhumi* varies in terrestrialness with the location of its colony and with the season. Nearly all *C. guanhumi* burrows extend down to ground water, on which crabs in inland colonies depend to replenish water losses. They must spawn in the sea, but otherwise they exist as land animals. Crabs living near water frequently enter it to feed, to avoid capture, and perhaps to migrate. In the low-lying coastal and estuarine areas which it inhabits, *C. guanhumi's* retention of amphibious ability and its independence of salinity greatly increase its food supply and provide protection from predators and climatic extremes.

A definitive estimate of *C. guanhumi's* ecological importance is beyond the scope of this paper. Its ability to starve for long periods complicates estimations of its food requirements. Even if these are low, the observation that its biomass approaches two metric tons/hectare in inland colonies, and may be higher along waterways, would seem to make it an important animal in both of these habitats.

Cardisoma is exceptionally accessible for observation. Its habits of daylight activity in inland colonies and its periodic daylight migrations, when hundreds or thousands of individuals may be visible at one time, make the occurrence of color and growth stages, sexual dimorphism, and an aberrant morphological minority readily apparent.

C. guanhumi also seems unusual among crustaceans in its lunar spawning behavior. In this context spawning is defined as the release of larvae from their attachment to the female; ovulation as the release of mature ova from the ovary, followed immediately by their fertilization, acquisition of a chitinous covering and attachment to pleopod hairs; maturation as the growth and yolk accumulation of ova in the ovary.

Lunar swarming in the prawn *Anchistoides antiguensis,* and lunar periodicity of color changes and motor activity in crabs have been reviewed or reported by Brown (1961a), Hauenschild (1960), and Bennett *et al.* (1957). None of these mention lunar spawning. Korringa (1947, 1957) cites many instances of lunar or semilunar spawning periodicity in polychaetes, molluscs, the grunion, *Leuresthes tenuis,* and the chironomid, *Clunio marina,* but does not mention lunar spawning in crustaceans. Calman (1911) quotes Andrews' and Stebbing's (reference not given) descriptions of spawning migrations by the gecarcinid crabs *Gecarcoidae lalandii* from Christmas Island, and *Gecarcinus ruricola* from the West Indies. In both species spawning is said to be simultaneous, *i.e.,* a mass spawning migration occurs, but this happens only once a year, during the rainy season. Andrews further stated that another Christmas Island gecarcinid, *Cardisoma hirtipes,* was not observed to enter mass spawning migrations.

Whether lunar spawning is rare in crustaceans or whether other forms have not been observed as closely as *C. guanhumi* is unknown, but its occurrence in this species seems reasonably certain. In the five-month 1961 spawning season 90% of all ovigerous females observed were seen in the six three-day periods preceding the full moons. A peak occurred one day before the full moon in the first four of these periods, and three days before in the last two. Smaller mid-period peaks also occurred.

Embryos taken from females migrating to the sea are generally near the same stage of development, implying a common ovulation date for most of the females spawning in a given period. It seems less likely that crabs can influence the rate of embryological development after the eggs are fertilized and attached to abdominal appendages, than that they can influence the rate of ovarian maturation or hold the mature ova to be released under the influence of a common external stimulus. Numerous instances of the latter in marine invertebrates are given by Giese (1959a, 1959b). Separate control (at least partly hormonal) of maturation and ovulation in *Uca pugilator* is suggested by the observation of Brown and Jones (1949) that eyestalk removal outside the normal spawning period is followed by ovarian maturation but generally not by normal ovulation and oviposition. Only one captive *C. guanhumi* has ovulated, and that was within a few days of capture. The simplest explanation of *C. guanhumi's* synchronized cyclic spawning consistent with these observations is that maturation is induced and roughly synchronized by a combination of internal conditions with one or more external factors, and that ovulation (hence spawning) is triggered by some external change occurring twice in each lunar cycle. Speculation on the nature of the external stimuli is not profitable in the absence of additional information.

Changes in overall coloration and shape with increasing size, similar to those occurring in *C. guanhumi*, have been reported for *Uca* (Crane, 1941a) and for *Ocypode* (Crane, 1941b; Cowles, 1906). Adults of both genera were said to have little shell pigment and to undergo physiological color change, either as a part of courting behavior (*Uca*) or in response to changes in background, illumination, or temperature (*Ocypode*).

The color change from blue to white or yellow, which occurs in ovulating *C. guanhumi*, appears similar to the morphological color change described by Brown (1934), in that the amount of yellow pigment in the epidermis increases. The gill chamber covering of *C. guanhumi* can be cut away with its epidermal lining intact. If the latter is stripped from the shell, much of the epidermal pigment remains behind. In deep blue crabs only black pigment can be obtained in this way, while in crabs ranging from blue-white to yellow, increasing amounts of yellow pigment appear. In completely yellow crabs the pigment adhering to the shell is almost all yellow, although much pigment can be rinsed from the epidermis. Completely yellow crabs may stay that way for months, without further change, but white crabs can undergo physiological color change. Ovigerous females are frequently blue or blue-gray when captured at night, but lighten in an hour or so if exposed to artificial light. They are almost invariably white or yellow the following morning.

The literature concerning hormonal control of physiological color changes is vast (Brown, 1961b; Kleinholz, 1961; Carlisle and Knowles, 1959), but very little has been done on morphological changes. It would seem that *C. guanhumi*, in which large numbers of crabs undergo such changes simultaneously at predictable times, would provide exceptional material for further study of them. The control of the various stages in the reproductive cycle might also be profitably investigated.

SUMMARY AND CONCLUSIONS

1. Observations on the general biology of the land crab, *Cardisoma guanhumi*, in southern Florida are presented, touching upon its geographical range, habitats, life cycle, growth, color changes and spawning and non-spawning migrations.

2. *C. guanhumi* may be nearly terrestrial, entering water only to replace water losses and to spawn, or it may be amphibious, entering either fresh or salt water to feed, to escape predators and climatic extremes, and perhaps to migrate.

3. Post-larval *C. guanhumi* pass through three growth stages, juvenile, transitional, and adult, which are defined on the basis of morphological and color changes.

4. A small minority (about 6%) of the population differs markedly in pigmentation, and to a lesser extent morphologically and in spawning behavior, from the normal.

5. *C. guanhumi* migrates to salt water to spawn in sharp lunar and semi-lunar peaks preceding the new and full moons between June and November. Non-spawning migrations, involving both males and nonovigerous females, sometimes follow the spawning migrations, but are less well-defined and may be localized.

6. Adult color is due to epidermal chromatophores. Females change from the normal adult blue to white or dull yellow at the time of ovulation. The overall change is morphological, involving production of yellow chromatophore pigment. Physiological color changes, absent at the extremes, can occur while this change is in progress.

7. Males may undergo similar color changes in autumn, but such changes are less frequent and usually incomplete.

8. Once attained, the yellow color is apparently retained through the fall and winter.

9. These observations are discussed, and some tentative explanations are given.

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TEMPERATURE AND OXYGEN CONSUMPTION OF ORCHOMONELLA CHILENSIS (HELLER) (AMPHIPODA: GAMMEROIDEA)

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O. chilensis was collected by several antarctic expeditions and probably is antarctic circumpolar (Shoemaker, 1945). The species has previously been divided into several forms or into several separate species; the antarctic populations were named *Orchomenopsis rossi* A. O. W. (Walker, 1903). Chilton (1912) concluded that *Orchomonella chilensis* is a widely distributed and variable species. It was originally described from Chile and has been collected from both the North and South Atlantic Oceans.

Walker (1907) reported *O. chilensis* was present in McMurdo Sound in enormous numbers from May through October and disappeared from the traps between October 25 and January 27, the time of the antarctic summer. However, the animals were readily collected in traps baited with seal meat and placed at a depth of 240 m. off Cape Armitage during the antarctic summer of December, 1960, and January, 1961.

Walker (1907) reported that this amphipod could exist only in water a little above freezing. Because of the recent studies of metabolic compensation in poikilothermic animals, it seemed of interest to measure the relationship between temperature and oxygen consumption of an animal that lives the entire year at a temperature near −1.8° C.

This research was supported by the National Science Foundation under Research Grant 13231. Mr. Hugh B. House assisted in the laboratory work. Dr. Gunther Schlager assisted with the statistical analysis of the data. The experiments were conducted in the Biology Laboratory, NAF, McMurdo, Antarctica.

MATERIALS AND METHODS

Animals collected from traps were returned to the laboratory in sea water in insulated cans. The animals were kept in aerated sea water in a constant temperature cabinet at −1.8° C. About 24–48 hours prior to a run, the animals were placed in a refrigerator or water bath at the same temperature as that of the run.

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However, because of the lack of facilities for temperature control, the animals were subjected to considerable variation in temperature at temperatures exceeding 2° C.

Wide-mouth bottles of about 60 cc. capacity were used as respirometers. Fresh, filtered sea water was aerated for 12 hours prior to a run. Sufficient water was stirred in a large beaker to fill 24 bottles. Three bottles were used as controls and one animal was placed in each of the remaining 21 bottles. All bottles were closed with a glass stopper and checked for air bubbles. The bottles were placed in a rack and the rack placed in a covered water bath of the proper temperature. Temperature of the water bath varied about 0.1° C. Most runs were 4 hours, but some were 6 or 8 hours.

At the end of each run, the amount of dissolved oxygen in each bottle was determined by means of the unmodified Winkler method. The difference between the amount of oxygen in a bottle with an animal and the mean of the three controls was the amount of oxygen used. Each animal was blotted dry and its wet weight determined on a Mettler Type B5 analytical balance. Because no brooding female amphipods were found, the sex of the animals was not determined.

TABLE I

Oxygen consumption and body size of Orchomonella chilensis (Heller)

Temperature, ° C.	Mean wet weight, nearest mg.	Oxygen consumption (μl./gm./hr.)		Coefficients					
				$O_2:W$			$\frac{O_2:W}{W}$		
		$\bar{x}$	S.E.$\bar{x}$	b	S.E.$_b$	r**	$b-1$	S.E.$_{b-1}$	r**
−1.8	67	128.3	0.17	0.455	0.103	0.408	−0.470	0.093	−0.455
0	87	117.7	0.15	0.471	0.058	0.631	−0.530	0.059	−0.675
2	86	141.2	0.16	0.462	0.106	0.403	−0.467	0.091	−0.459
4	75	124.3	0.16	0.665	0.083	0.630	−0.353	0.099	−0.339
6	71	146.8	0.17	0.642	0.107	0.519	−0.309	0.108	−0.278
8	71	158.8	0.14	0.604	0.061	0.707	−0.393	0.061	−0.543
10	70	231.0	0.10	0.571	0.078	0.596	−0.448	0.062	−0.590
12	75	176.8	0.15	0.497	0.069	0.587	−0.589	0.090	−0.553

All values of r highly significant. Test for heterogeneity of r: null hypothesis, all r's are from the same population. For $O_2:W$, $\chi^2 = 16.92$, d.f. = 7, $.02 > P > .01$. Reject null hypothesis. $\frac{O_2:W}{W}$, $\chi^2 = 20.57$, d.f. = 7, $.01 > P > .001$. Reject null hypothesis.

A preliminary survival experiment indicated that the animals were intolerant to temperatures above 15° C. Therefore, runs were made at −1.8° C., 0° C. and at 2° intervals through 12° C. At 12° C., there was considerable mortality in the respirometers and it was not feasible to make determinations of oxygen consumption at higher temperatures. The oxygen consumption of 100 animals was determined at each temperature; 800 animals were used overall.

Two sources of error should be noted. No attempt was made to control activity. There was no control over the amount of feeding by the animals except they had not fed for at least 24 hours prior to being used. In addition, the animals were not acclimated to the experimental temperatures. Thus the R-T curve is acutely measured. Probably the animals lived for several months at −1.8° C. or slightly warmer before they were brought into the laboratory.

Oxygen Consumption and Size

The consumption of oxygen per unit time is a function of size as expressed in the following equation:

$$(1)\quad O_2 = aW^b,$$

where W = weight and a and b are constants. Equation (1) may be divided by W to produce a weight-specific respiration:

$$(2)\quad \frac{O_2}{W} = aW^{b-1}.$$

In these equations, ^{b} and $^{b-1}$ are regression coefficients. For crustaceans, ^{b} is generally between 0.67 and 1.0 and $^{b-1}$ is usually between −0.05 and −0.40 (Wolvekamp and Waterman, 1960).

The regression coefficients of double-logarithmic plots were determined by the method of least squares. Additional statistics calculated were the standard errors of ^{b} and $^{b-1}$ and the coefficients of correlation (r) (Table I). A different ^{b} (Fig. 1) or $^{b-1}$ was obtained at each temperature. Because the standard errors were so variable, the regression lines of ^{b} were tested for homogeneity by an analysis of covariance (Steel and Torrie, 1960; p. 319). The null hypothesis, there is no difference in regression coefficients for log oxygen consumption against log weight at the 8 temperatures, was tested by means of the F test. Because $F = 5.407$ ($p < .01$), the null hypothesis was rejected and it was concluded that the regression lines were different from one another. However, there was considerable heterogeneity in the unexplained SS at 8°, 10° and 12° C. Therefore, a second test for homogeneity was made, omitting the three higher temperatures. Because $F = 5.66$ ($p < .01$), it was concluded that the regression slopes were heterogeneous.

Heterogeneity in regression slopes was found in *Artemia salina* (Conover, 1960), in the snails *Lymnaea palustris* and *L. pereger* (Berg and Ockelmann, 1959) and in the crabs *Uca pugnax* and *U. rapax* (Vernberg, 1959). Vernberg and Conover demonstrated a direct effect of temperature on ^{b} or $^{b-1}$. Berg and Ockelmann demonstrated a seasonal shift in ^{b}; when the animals were tested at 18° C., the slope of the regression line was greater in June than in August. The authors suggested that the seasonal variation might be caused by a comparatively greater increase of oxygen consumption by the larger animals during the season of reproduction. However, in this regard, Rao and Bullock (1954) showed that habitat temperature of the animal prior to study could affect Q_{10} and that Q_{10} commonly increases with increasing size over the range of normal physiological activity. Presumably the differences in habitat temperatures might account for the differences reported by Berg and Ockelmann, although it is not unlikely that several interacting factors were present.

Temperature seems more likely to be the primary factor in explaining the variation of ^{b} in *O. chilensis*. The animals were collected from an environmental temperature that probably varied less than 1° C. for several months, nor were the animals reproducing. Although Q_{10} was not calculated, it is evident from Figure 2

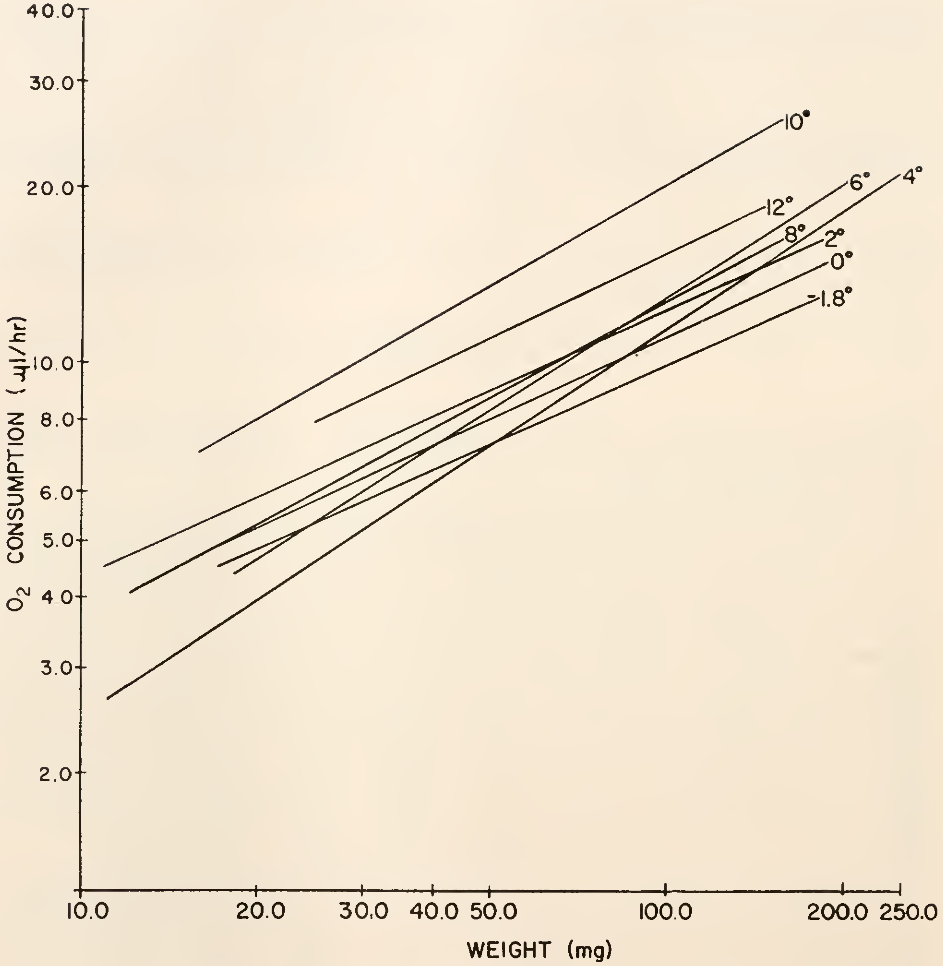

FIGURE 1. The relationship between oxygen consumption and body size in *Orchomonella chilensis* (Heller). Temperature is degrees Celsius (C).

that Q_{10} varied with size. Small animals greatly reduced oxygen consumption at 4° C. as compared with 2° C. With increasing size, the amount of reduction decreased and the largest animals increased consumption. Another trend appeared between 6° and 8°, the smallest animals increasing and the largest animals decreasing consumption.

These trends are supported by the values of r. Because the distribution of r's is asymmetrical, all r's were converted to the normally distributed z and the z's tested for homogeneity. The null hypothesis, all r's are from the same population, was rejected (Table I). Therefore, it is evident that the relationship, O_2:W, is influenced by temperature. The z's were tested for significant differences (Table II). Significant differences, among others, occurred between 2° and 4° C. and

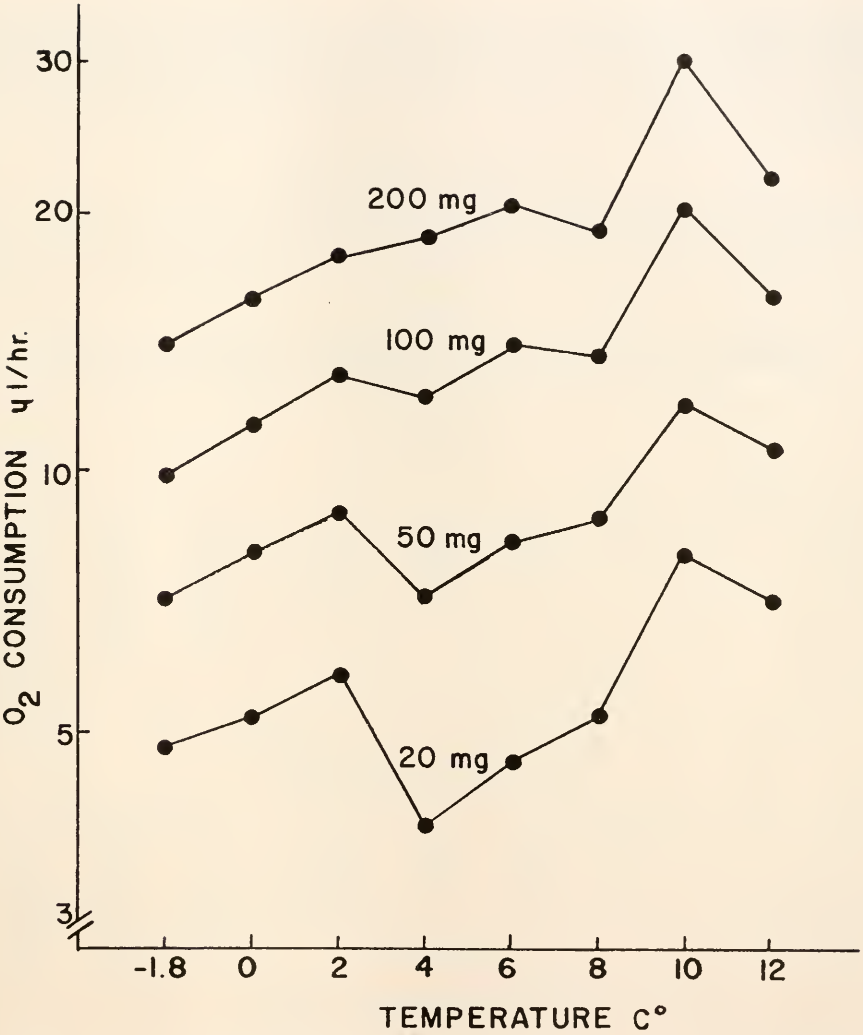

FIGURE 2. The relationship between oxygen consumption and temperature for different sized *Orchomonella chilensis* (Heller).

between 6° and 8° C. Similar results were obtained comparing the *r*'s of the relationship $O_2/W:W$. However, the distribution of significant differences among *z*'s of the weight-specific rate:weight values differed from the distribution of significant differences among *z*'s of the rate:weight values. Although the general conclusion that temperature affects the rate:weight relationship does not depend on the method of evaluating rate, the specific effects of temperature do depend on the method of analysis.

According to von Bertalanffy and Krywienczyk (1953), if $^b = 0.66$, metabolism is proportional to surface; if $^b = 1.0$, metabolism is proportional to weight; if

$^b > 0.66$, but < 1.0, metabolism is intermediate. The values of ^{b} for *O. chilensis* indicate several metabolic types in the population including types not included in the above scheme. Locker (1961), however, believes that metabolism is basically related to surface, but this relationship can be modified by factors such as temperature.

RATE-TEMPERATURE CURVE

The shape of the R-T curve (Fig. 3) will depend, in part, on the size of the animals used and will reflect the composition of the population at the time the animals were collected. For example, oxygen consumption was higher at 0° C. than at −1.8° C. for each size class (Fig. 2), but the rate at 0° C. was lower than at −1.8° C. (Fig. 3) because the population at −1.8° C. averaged 20 mg. less in size (Table I). However, the decrease of oxygen consumption at 4° C. occurred in all except the largest members of the population (which were rare) and therefore seems characteristic of the population in general. This decrease appears to be an adaptation to high temperatures. The adaptation lies in the vertical displacement of the R-T curve and suggests a change in Q_{10}. This change might be brought about by a shift in the control of metabolic reactions to an alternate enzymatic pathway.

TABLE II

Differences between r's converted to z. All differences exceeding the LSD marked with an asterisk

Rate: Weight ($O_2:W$)

° C.	−1.8	0	2	4	6	8	10	12
−1.8	0	.310*	.006	.308*	.142	.448*	.254	.240
0		0	.316*	.002	.168	.138	.056	.070
2			0	.314*	.148	.454*	.260	.246
4				0	.167	.140	.055	.068
6					0	.306*	.112	.098
8						0	.194	.208
10							0	.014
12								0

Weight-specific Rate: Weight $\frac{(O_2:W)}{W}$

° C.	−1.8	0	2	4	6	8	10	12
−1.8	0	.329*	.005	.137	.206	.117	.187	.131
0		0	.324*	.466*	.534*	.212	.142	.198
2			0	.142	.211	.112	.182	.126
4				0	.069	.254	.324*	.268
6					0	.323*	.392*	.336*
8						0	.069	.013
10							0	.056
12								0

Significant differences among z's: $t = z_1 - z_2 \Big/ \sqrt{\frac{2}{n-3}}, = 0.282.$

Following the shift between 2° and 4° C., the rate of oxygen consumption increased and beyond 8° metabolic control was inadequate. Under acclimated conditions, the animals might be fairly good regulators to 6° or 8° C., but would likely be conformers in any event at temperatures above 8° C. One might not expect such a high degree of regulation in a species living at such a narrow range of environmental temperature. It would be interesting to determine the response pattern of the species in other parts of its geographical range, as the R-T curve of the antarctic population may result from the genetic history of populations that previously lived in waters with wider fluctuations of temperature than now seem to occur in McMurdo Sound.

The levels of oxygen consumption were about the same as arctic amphipods (Scholander *et al.*, 1953) and were higher than in amphipods of temperate regions (Wolvekamp and Waterman, 1960), when the differences in experimental temperatures are eliminated by extrapolating the curves of temperate species downward or projecting the slope between 8° and 10° of *O. chilensis* upward. Thus, *O. chilensis* clearly demonstrates metabolic compensation (Bullock, 1955).

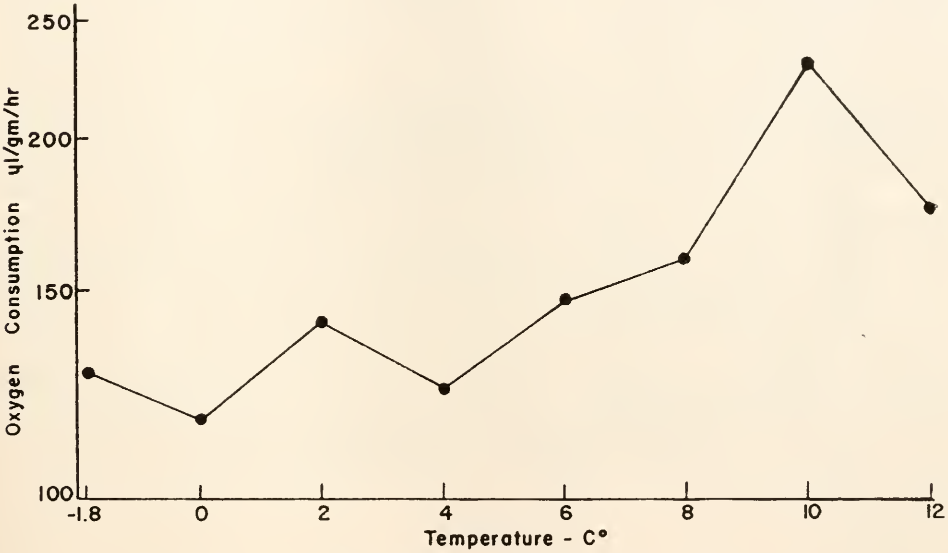

FIGURE 3. The acutely determined R-T curve for populations of *Orchomonella chilensis* (Heller).

SUMMARY

1. The rate of oxygen consumption of the antarctic amphipod *Orchomonella chilensis* (Heller) was determined over a graded temperature series from −1.8° to 12° C.

2. The regression coefficients of double-log plots of rate:weight and weight-specific rate:weight were temperature-dependent. The correlation coefficients

between size and rates of consumption were highly significant and varied significantly with temperature. Q_{10} varied with size.

3. The acutely determined R-T curve shows some regulation between −1.8° and 6° C. Metabolic compensation was evident.

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THE ANALYSIS OF POLARIZED LIGHT IN THE EYE OF DAPHNIA[1]

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The operation of any polarization detector involves: (1) separation of incident light into two vectors perpendicular to each other and to the direction of propagation of the light beam, (2) suppression of one vector, (3) intensity estimation of the remaining vector. In physical instruments polarization analysis depends on rotation of the analyzer around the direction of propagation of the light beam. In the biological systems found in arthropods, polarization analysis depends on the possession of a radial array of analyzers, whether this is the rhabdomere-retinula cell complex or the many different corneal and lens surfaces of the compound eye of arthropods. Such an array of analyzers permits simultaneous comparison of polarized intensities present at all azimuths about the direction of propagation of the light beam. These comparisons may be between different ommatidia, with the receptor system of each ommatidium acting as a unit, or between parts of the receptor system of a single ommatidium.

Three models have been proposed to account for the orientation of animals to polarized light, of which two are intra-ocular, the third extra-ocular. The three models are: (1) the Brewster-Fresnel model in which one or more refractions and reflections at corneal or lens surfaces serve to alter preferentially the intensity of light polarized parallel to the plane of incidence; (2) dichroic filters (the rhabdomeres) with the fast axes tangential to the radii of the array of filters; (3) the reflected brightness pattern in which the intensity of light reflected or scattered from the environment is greater perpendicular to the light polarization plane.

The Brewster-Fresnel refraction model relying upon a single refraction was proposed by Stephens, Fingerman and Brown (1953) for the *Drosophila* eye. The Brewster-Fresnel reflection model relying upon internal reflection from a lenticular surface was proposed by Baylor and Smith (1953) for *Daphnia*. The presence of dichroic filters in the eye of the bee was suggested by Autrum (see von Frisch, 1950) and has been supported by Stockhammer (1956, 1959). The environmental reflection pattern as an orientation stimulus was suggested by Baylor and Smith (1958).

Values of the theoretical light intensities calculated from the Fresnel equations are compared with actual measurements through two surfaces of the daphnid cone lens. The data presented here support the first of these intra-ocular models for the eye of *Daphnia pulex* (de Geer).

MATERIALS AND METHODS

The measurements were made on the lenses of freshly killed daphnids mounted in water under a coverslip on a microscope slide and examined at 500 × under a

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Leitz Ortholux microscope with a trinocular head. A rotatable type N Polaroid filter was interposed in the light beam beneath the microscope condenser. A 10 × ocular fitted with a field stop diaphragm replaced the camera on the trinocular head and was coupled to a photomultiplier photometer (Photovolt model No. 501M) for measuring light intensities. The field stop diaphragm placed at the image plane of the ocular restricted the field of view of the photomultiplier to a circle 10 μ in diameter. The center of the circle was coincident with the intersection of crosshairs in one of the viewing oculars to permit location of the desired area.

The change in light transmission through the carapace adjacent to the eye was measured as the Polaroid was rotated through 90° from a position parallel to the preferred transmission plane of the microscope to one perpendicular to that plane. Individual lenses were aligned with the preferred transmission plane of the microscope and the change in transmission of refracted light was again measured with the Polaroid in two positions. These measurements were repeated with the long axis of the lens rotated 90°.

RESULTS AND DISCUSSION

The measurements from 24 cone lenses from 6 different eyes are summarized in Table I. Columns 2 and 5 contain data based on the measured intensities of light transmitted through the carapace alone. These percentages are calculated by dividing the reading of the photometer with the Polaroid in the east-west position by the reading with the Polaroid in the north-south position (NS/EW × 100), where EW is the preferred transmission plane of the optical system of the microscope. This always gives a calculated value of less than 100. A comparison of columns 2 and 5 shows the difference due to the carapace alone, because the carapace has been turned through ninety degrees. The differences measured in this manner are small, and are probably random, indicating the carapace is not an effective polarization analyzer. A comparison of columns 1 and 2, and columns 5 and 6 shows not only the difference between the effectiveness of the lens and the effectiveness of the carapace but also that the preferred transmission plane of the hemispherical lens is parallel to the long axis of the cone. In columns 2 through 4, with the lens oriented NS, the average transmission of the background, 77.9%, is exceeded by that of the lens, 81.3%, a difference of 3.4%. This is because the lens transmits a greater proportion of the light when the Polaroid is in the NS position, that is, parallel to the lens axis. In columns 5 through 7 the reverse is true: the lens faces EW, its preferred plane is EW and therefore the fraction NS/EW becomes smaller when the Polaroid is turned from EW to NS, that is, perpendicular to the lens axis. The mean observed change is from 77.3% to 74.2%, a difference of 3.1%. Columns 4 and 7, taken by themselves, measure the change in intensity corrected for the lens system of the microscope, with the difference in column 4 being positive, that in column 7 negative. Of the 48 readings, four differ in a direction opposite to the expected direction. The mean difference of all the measurements, correcting for the difference in sign, is 3.25% and lies between 2.0% and 4.5% with a probability greater than .99. This difference is consistent with the calculated values for the Brewster-Fresnel reflection model. Reference to Figure 1 shows that measurements have been made on light diffracted both at point B and at point D.

EW/NS ,100

TABLE I

Measured values of light intensity

	Lens NS			Lens EW		
	% transmission of background	% transmission of lens	Difference	% transmission of background	% transmission of lens	Difference
1	81.00	83.75	2.75	76.50	71.59	−4.91
2	76.50	85.00	8.50	77.00	75.00	−2.00
3	77.00	82.24	5.24	77.00	73.74	−3.26
4	77.00	80.62	3.62	76.50	72.83	−3.67
5	78.00	96.67	18.67	77.00	75.38	−1.62
6	77.70	78.57	0.87	77.50	73.38	−4.12
7	80.00	82.02	2.02	77.00	73.38	−3.62
8	76.00	82.43	6.43	77.50	74.52	−2.98
9	76.00	83.78	7.78	78.50	72.29	−6.21
10	77.00	80.72	3.72	78.00	75.66	−2.34
11	78.00	80.49	2.49	77.50	69.87	−7.63
12	77.00	81.32	4.32	77.00	73.97	−3.03
13	79.00	79.89	0.89	78.50	75.29	−3.21
14	78.00	81.71	3.71	78.00	74.29	−3.71
15	78.00	80.00	2.00	78.00	73.54	−4.46
16	76.00	75.00	−1.00	78.00	75.34	−2.66
17	79.00	82.56	3.56	78.50	74.26	−4.24
18	79.00	78.87	−0.13	77.50	78.31	+0.81
19	77.00	79.76	2.76	75.50	74.71	−0.79
20	76.00	76.68	0.68	78.50	75.58	−2.92
21	78.00	83.33	5.33	78.50	76.37	−2.13
22	77.00	77.95	0.95	77.00	76.16	−0.84
23	82.00	77.78	4.22	77.00	72.63	−4.37
24	80.00	81.08	.108	74.00	73.85	−0.15
Mean	77.92	81.34	3.42	77.33	74.25	−3.08

Table II summarizes the calculated intensities for rays incident between 10° and 80° on the external surface of the daphnid cone lens (angle Ø in Figure 1) and whose plane of polarization is either parallel or perpendicular to the long axis of the lens. For these calculations the index of refraction is assumed to be 1.53 and that of the blood 1.33, yielding a relative index of refraction of 1.15. Since the tangent of the angle of maximum polarization equals the relative refractive index, this angle is 49° for the external surface and 41° for the internal surface. To simplify the optical calculations and discussion, only those rays confined to a single plane are considered throughout the paper. This plane bisects the conical figure of the lens and is identical with the plane of incidence, the latter of which is defined by the incident ray and the perpendicular at the point of incidence. As is customary, the polarization plane is described as either parallel or perpendicular to the plane of incidence. For example, AB parallel means the light beam AB, which is parallel to the plane of incidence at B in Figure 1. In Table II, columns 2 through 7 contain values of reflected and refracted intensities corresponding to the labeled portions of the light paths in Figure 1. These values are referred to an intensity of 100 in the incident ray, AB.

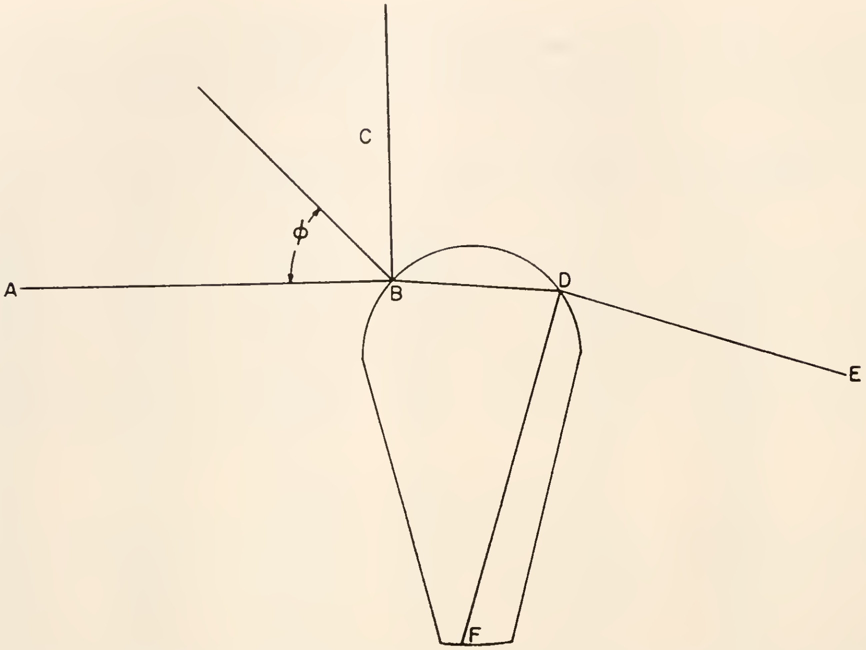

FIGURE 1. Diagram of a single cone lens showing the light path ABDF for the Brewster-Fresnel internal reflection model. Light from A is incident at B with the angle of incidence labelled Ø.

Columns 2 and 3 show the small differences which exist after the initial reflection and refraction at B; columns 4 and 5 give the somewhat greater differences occurring after two refractions at B and at D. The differences between DE parallel and DE perpendicular are given in column 9 and correspond to the measured differences of Table I. The maximum *calculated* difference at 70° is 3.28% while the mean *observed* difference is 3.0%. That these values are in such good agreement is

TABLE II

Calculated values of light intensities

1 Angle of incidence	2 $BD_{\perp}$	3 $BD_{\parallel}$	4 $DE_{\perp}$	5 $DE_{\parallel}$	6 $DF_{\perp}$	7 $DF_{\parallel}$	8 $DF_{\perp}/DF_{\parallel}$	9 $DE_{\perp}$-/$DE_{\parallel}$
10	86.16	86.21	74.24	74.32	0.45	0.40	1.125	00.08
20	85.02	85.20	72.28	72.59	0.52	0.32	1.625	00.31
30	82.90	83.34	68.72	69.46	0.66	0.21	3.143	0.71
40	79.34	80.24	62.96	64.38	0.95	0.07	13.57	1.44
50	73.36	74.89	53.82	56.09	1.51	0.15	10.03	2.27
60	63.26	65.72	40.02	43.20	2.65	0.30	8.32	3.18
70	46.26	49.68	21.40	24.68	4.73	1.77	2.67	3.28
80	20.43	24.53	4.17	06.02	6.13	4.80	1.27	1.8

The value of the incident ray, AB, is 100.

largely chance because any single measurement in Table I gives an average value for many degrees of incidence. The fact that any difference whatsoever can be shown in Table I probably means that (1) the index of refraction is higher than the

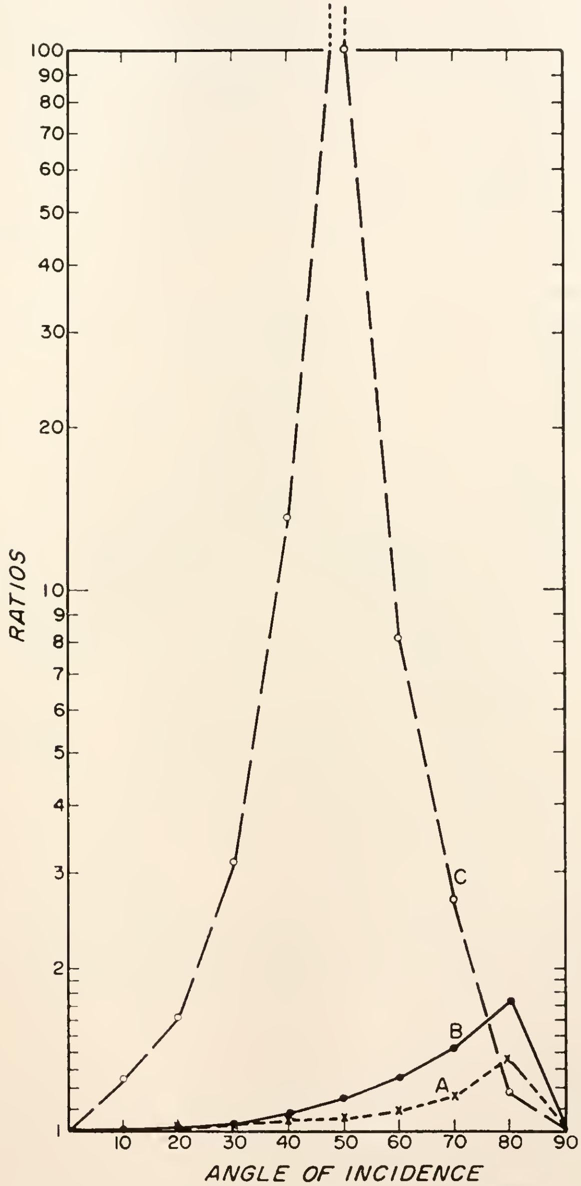

FIGURE 2. Ratios of vector intensities resolved at corneal interfaces. Curve A is $DE_{\parallel}/DE_{\perp}$. Curve B is calculated from data of Stephens, Fingerman and Brown (1953) and corresponds to $BD_{\parallel}/BD_{\perp}$ for a cornea-air interface. Curve C is $DF_{\perp}/DF_{\parallel}$. The subscript $\perp$ means the ray is polarized perpendicular to the plane of incidence and the subscript $\parallel$ means the ray is polarized parallel to the plane of incidence.

assumed value of 1.53 and (2) the optical surfaces of the cone lens are not hemispherical and therefore the overall efficiency of analysis is higher. A more effective polarization analyzer could be realized by altering the shape of the optical surfaces so that more of the incident rays would meet the surface close to the polarizing angle. The shapes of cone lenses will be discussed later.

The effectiveness of the resolution of the refracted ray into two vectors is shown in Figure 2, where curve A is the plot of log (DE parallel/DE perpendicular). Contrasted with this is the vector resolution of the reflected ray, curve C, log (DF perpendicular/DF parallel), where the ratio of vector intensities is very high, especially near the polarizing angle. At the polarizing angle internal reflection of the parallel ray diminishes to zero and therefore the ratio approaches infinity. In this way the small differences which were measured for *Daphnia* can be associated with an effective analysis within the eye. It should be emphasized, however, that the absolute intensity of rays at the receptor in this model is low.

The effectiveness of any light polarization analyzer depends upon its ability to separate the incident light into two vectors and to present these two vectors for intensity measurement. The ratio of the intensities of the two vectors is thus a measure of the effectiveness of the polarization analyzer. The calculations in Table II permit a comparison of the effectiveness of the two different kinds of Brewster-Fresnel models which have been proposed. The first model depends upon a single refraction at the corneal surface and was proposed for the eye of *Drosophila* by Stephens, Fingerman and Brown (1953). The effectiveness of this model in resolving the incident light into two vectors depends on a high relative index of refraction characteristic of an air-cornea interface but not of a water-cornea interface. The ratios of the different vector intensities resolved by a single refraction at the cornea-air interface and by two refractions at the cornea-water interface are shown in curves A and B of Figure 2. Comparison of these two curves with curve C of the same figure shows unequivocally that refraction is not as effective as reflection for polarization analysis. It remains to be seen whether beams of light incident on the terrestrial arthropod cornea at high angles proceed by multiple reflection to the light-sensing apparatus. A sequence of such internal reflections (at less than the critical angle) would be a very effective light polarization analyzer. The observations of de Vries and Kuiper (1958) for Diptera and Waterman (1954) for *Limulus,* that ommatidia are sensitive to light incident at high angles, might be thought of as lending credence to this view. However, Waterman's (1954) work relating intensity threshold to angles of incidence raises serious doubts concerning the Brewster-Fresnel refraction model in the natural habitat because the intensity threshold for light incident near the polarizing angle is approximately 100 times greater than that of light normal to the surface. The confusion of polarized intensities with non-polarized intensities and the obscuring of any particular polarized light stimulus seem inevitable with this model unless this eye possesses an ability to distinguish 1% brightness differences.

The second Brewster-Fresnel analyzer model proposed by Baylor and Smith (1953) involves the somewhat unorthodox light path ABDF of Figure 1, which requires the light to be incident at the cornea-blood interface twice, once on entering at B and again on being reflected at D. The ratios of orthogonally polarized intensities reflected from D are plotted in curve C of Figure 2 where they give a somewhat exaggerated impression of the effectiveness of this light polarization analyzer when the ratio of intensities goes to infinity at the polarization angle. The operation of this model may be seen in three dimensions in Figure 3. In Figure 3 the cone lenses are depicted on xyz coordinates to represent a solid figure. A ray of light parallel to the y axis and polarized parallel to the z axis is incident on the surmounting hemisphere of each of the cone lenses. The intensities resulting from subsequent refractions and reflections are summarized on the figure and were taken from the 60° line of Table II where the ratio of the intensities at the light-sensing apparatus is approximately 8 to 1.

Microscopic observations of the compound eye of *Daphnia pulex* reveal that the cone lens is not a circular solid cone of 45° surmounted by a hemisphere. Considerable variation in shape and contour is observed in the lenses of the eyes studied. In particular, one type of cone lens has a rather special shape in which the contours exhibited are of considerable theoretical interest because they are comparable to those predicted and drawn on paper from simple geometrical optical considerations. Starting with the knowledge that the light-sensing apparatus lies at the tip of the cone lens and with the constraint that the angle of the cone should be approximately 45° we may reconstruct the light path FDBA of Figure 1 through the cone lens step by step, starting at the apex and working backward to the outside

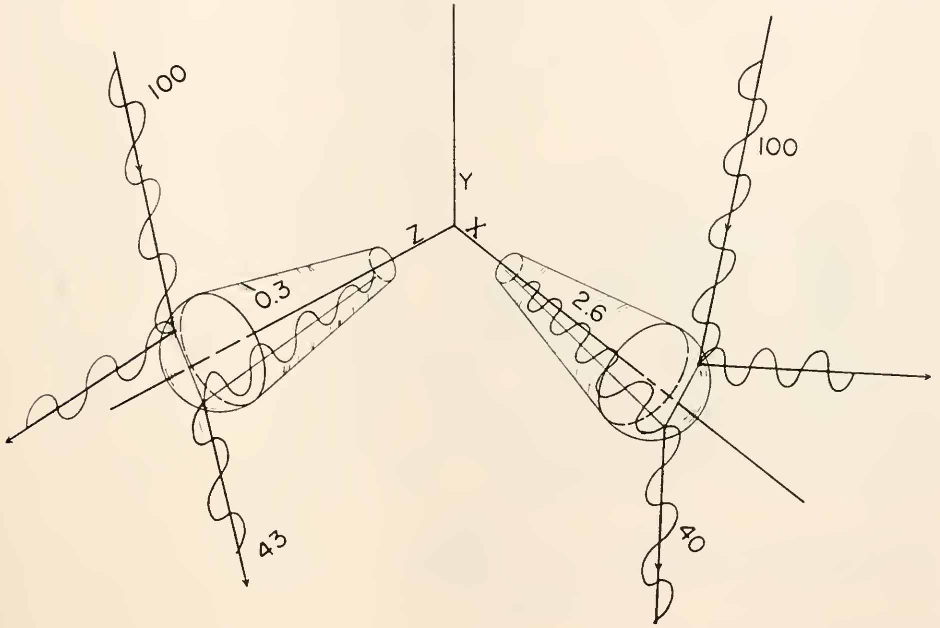

FIGURE 3. Three-dimensional diagram of the Brewster-Fresnel internal reflection model showing two cone lenses at right angles. Light rays are incident parallel to the y axis with an intensity of 100, and polarized parallel to the yz plane. The numbers represent calculated intensities at the various parts of the light path.

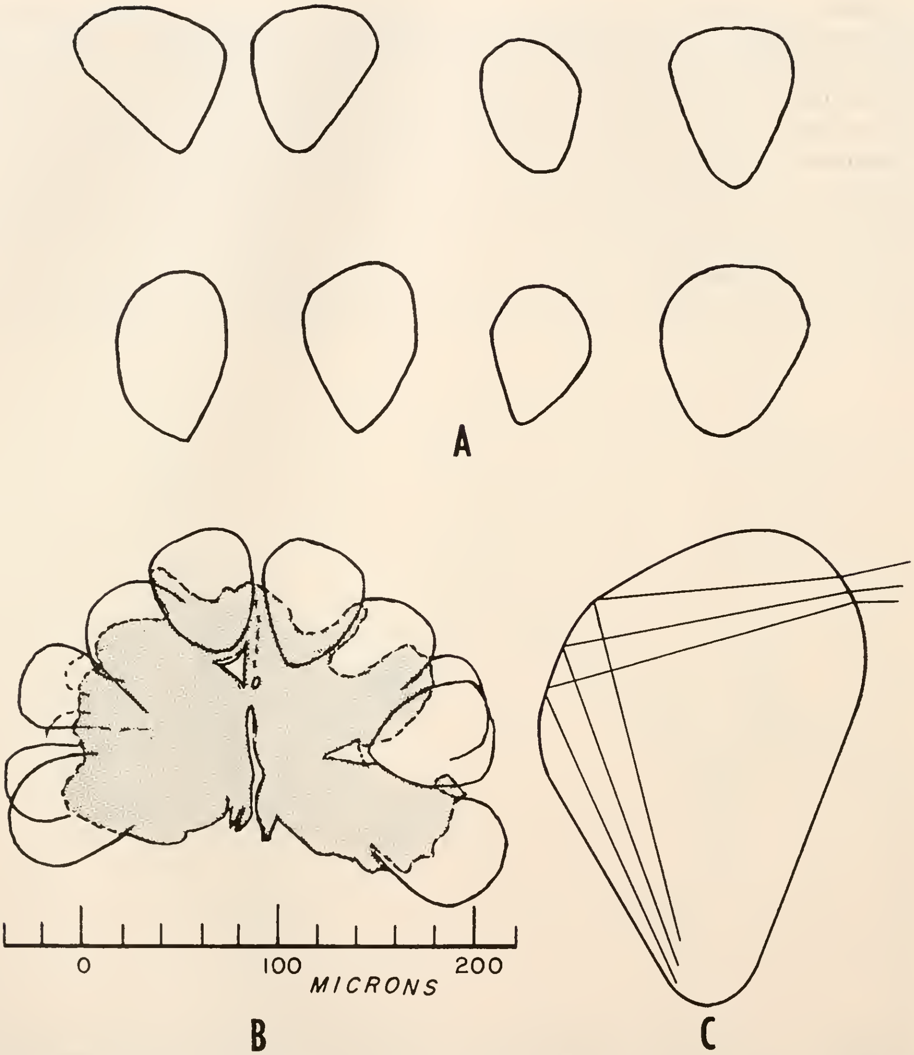

FIGURE 4. A, Outlines of selected cone lenses of *Daphnia pulex*. B, Composite tracing of photographs of three serial frontal sections through the compound eye of *Daphnia pulex*. Optic nerve protrudes from the center toward the 100-micron mark of the scale. C, Constructed lens with three light rays.

of the lens. A series of rays 5 to 10° apart are drawn from the apex toward the open end of the cone. For maximum efficiency of polarization detection each of these rays should be reflected from the periphery of the lens at the polarizing angle. Therefore, at the open end of the cone we construct across each ray a line which intersects the ray at this polarizing angle. The distances along the rays from the apex to the intersections are adjusted so that the constructed lines intersecting the rays produce a smooth curve. At each intersection of a ray with the curve so produced a line is drawn perpendicular to it to permit construction of the reflected ray DB of Figure 1. The reflected rays are extended across the long axis of the cone toward B. A second intersecting surface is constructed across the rays to form a smooth curve which refracts all rays outward into a parallel bundle. The two smoothed theoretical surfaces are then joined across the base of the cone to complete the constructed figure. The completed figure (Fig. 4 C) cannot be superimposed on any photographs of cone lenses (Fig. 4 A and B) but provides a better approximation to the actual figure than does a hemisphere. These observations are consistent with the hypothesis that the dioptric contours of some cone lenses are specialized for polarizing angle reflection of light beams traveling at right angles to the long axis of the cone. The observations are also consistent with the intensity ratios measured in the light beam DE of Figure 1 and summarized in Table I, which are higher than anticipated from the calculations in Table II. The observed dioptric contours may also serve to decrease the intensity of ambient light incident parallel to the long axis of the cone. Figure 4 shows a constructed lens with outline drawings of selected lenses. Changes of lens shape as a result of fixation appear to be small when photographs of fixed material are compared with those of living material.

It is difficult to see how any of the models for light polarization plane detection operate effectively in a natural situation where the intensity of polarized light with a particular direction of propagation incident upon a receptor is masked by and confused with the intensity of light, whether polarized or non-polarized, from all other sources. When this happens the receptors must be able to distinguish intensity differences of a few per cent if orientation is to be precise. If we assume the model to be a perfect detector in the sense that the NS detector receives all light polarized in the NS plane and rejects all light polarized in the EW plane, it is still subject to confusion by ambient non-polarized light. Even with 100% polarized light the intensity ratios present for comparison in the Brewster-Fresnel external reflection model of Stephens *et al.* are not as great as 2:1, whereas the Brewster-Fresnel internal reflection model and the Autrum model have a maximum theoretical intensity ratio of infinity. The Brewster-Fresnel internal reflection model proposed for the daphnid cone lens appears especially vulnerable to the criticisms outlined above because such a small percentage of the incident polarized light is transmitted to the light-sensing apparatus. Therefore, it might be assumed that the reflected brightness pattern is the sole orienting stimulus. That it is not has been shown by Baylor and Smith (1953) and by Waterman (1960) who showed that orientation remained in spite of careful filtration of the water. In a separate experiment Smith and Baylor (1960) used a small half-wave plate umbrella to rotate the polarization plane of only the light directly incident on the daphnid without altering the reflection pattern. Here the daphnid oriented only to the polarized light plane incident from overhead unless the water was deliberately made turbid by addition of yeast. The function of polarized light responses in nature remains to be demonstrated and the possibility should not be ignored that many cases of polarized light responses may be only laboratory curiosities.

We wish to acknowledge the contributions of Prof. Frederick E. Smith of the University of Michigan with whom studies on the geometric optics of *Daphnia*

magna lenses were begun. Also, we wish to acknowledge a similar set of calculations by G. Schreuder-van Zanten and J. W. Kuiper in a manuscript sent to us by Prof. Kuiper.

SUMMARY

1. Three models suggested to account for the ability of arthropods to detect the plane of linear polarized light are characterized.

2. Measurements of polarized light refracted through the cone lens of *Daphnia pulex* are summarized.

3. These measurements are compared with calculated intensities derived from one of the three models.

4. The shape of the cone lens of *Daphnia* and the specialization of their contours for polarization analysis are suggested.

5. The operation of the various models in nature is criticized.

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BEHAVIOR OF DAPHNIA IN POLARIZED LIGHT[1]

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Three models have been proposed to account for the apparent ability of animals to perceive the plane of vibration of polarized light. Two of the proposed models are intra-ocular, the third is extra-ocular. The three models are: (1) a radial array of dichroic filters (rhabdomeres) with their fast axes tangential to the radii of the array; (2) the Brewster-Fresnel models in which one or more refractions and reflections at corneal or lens surfaces serve to diminish preferentially the intensity of light polarized parallel to the plane of incidence; (3) the reflected brightness pattern in which the intensity of light reflected and scattered from the environment is least parallel to the polarization plane and greatest perpendicular to the polarization plane.

Two Brewster-Fresnel models have been proposed. The Brewster-Fresnel reflection model relying upon a single refraction was proposed by Stephens, Fingerman and Brown (1953) for the *Drosophila* eye. The Brewster-Fresnel reflection model relying upon internal reflection from a lenticular surface was proposed by Baylor and Smith (1953) for *Daphnia.* That daphnids utilize an intra-ocular analyzer in clear water was established by Baylor and Smith (1960) using half-wave plates to distinguish between intra-ocular and extra-ocular polarization analyzers. These experiments corroborated their earlier findings (Baylor and Smith, 1953) as well as those of Waterman (1960). To test the Brewster-Fresnel internal reflection model, Baylor and Hazen (1962) conducted optical analyses of the lenses of *Daphnia pulex* (de Geer), including a microphotometric study of polarized light transmitted by the lenses. Their results are in agreement with the Brewster-Fresnel internal reflection model. The present paper examines the consequences of this model on the behavior of daphnids under polarized light.

We assume that in its response to polarized light, the daphnid moves so that the rhabdomeres of the forward ommatidia receive maximum light intensity. If this assumption is true, then the addition of light to the lateral ommatidia should disrupt the precision of the daphnid response to the polarization plane. The degree of disruption should be proportional to the amount of light added to the lateral ommatidia.

For polarization detection, the intensity of light at the rhabdomeres is maximum when the polarization plane of the incident light is perpendicular to the long axis of the cone. The forward and lateral ommatidia are perpendicular to each other in a horizontal plane, and therefore present mutually perpendicular planes of incidence to a vertical beam of light, as in Figure 1. The Fresnel equations require that whenever the forward-directed ommatidium has a maximum intensity at the rhabdomeres, then the laterally directed ommatidium has a minimum intensity at its rhabdomeres; the ratio of the intensity is approximately 8:1. Experimentally changing this ratio by directing horizontal light beams at the lateral ommatidia of a population of daphnids already orienting to a vertical beam of polarized light should produce an orientation in which the number of animals directed to the stimulus of the lateral beam is the same as the number directed by the vertical beam.

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We report here three sets of experiments. The first, with nonpolarized light, shows how a population of *Daphnia* oriented to two horizontally opposed light beams (AB in Fig. 2) changes orientation upon the addition of a second pair of horizontally opposed light beams (CD in Fig. 2) perpendicular to the first. This experiment tests the validity of the primary assumption on which the Brewster-Fresnel internal reflection model rests, *i.e.*, that positive phototaxis is guided by maximum intensity reception in the forward ommatidia. The second set of experiments shows how the orientation to a vertical polarized beam is altered by a pair of horizontally opposed beams parallel to the plane of polarization; in this experiment the lateral beam illuminates the lateral ommatidia of those animals responding to the plane of polarization. The third set of experiments shows how daphnids which appear to be primarily photonegative nevertheless have a secondary, weaker positive phototaxis which operates at right angles to the primary and vigorously negative phototaxis.

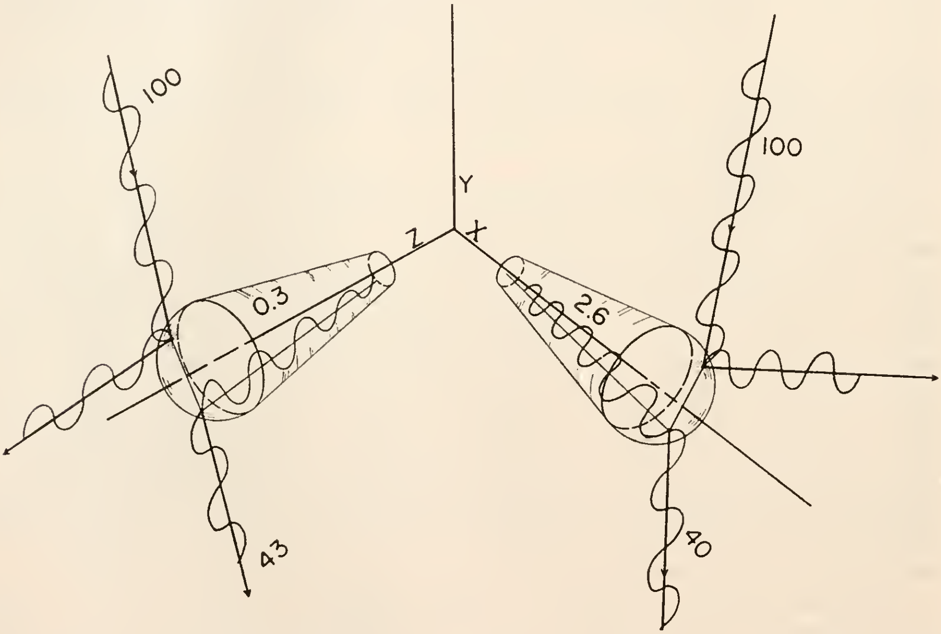

FIGURE 1. Three-dimensional diagram of the Brewster-Fresnel internal reflection model, showing two cone lenses at right angles. Polarized light is incident from above with an incident value of 100. The plane of polarization is parallel to the YZ plane. Numbers represent intensities at various parts of the light paths.

PROCEDURE

Experimental animals were from a laboratory culture of *Daphnia pulex* (de Geer) grown under constant light and fed a mixture of algae and yeast daily. Approximately a hundred of these animals were placed in filtered water in a lucite tank one foot on each side and shielded from stray light in a darkened room. A projection lamp with lenses and a polarizer hung four feet above the tank and provided a linearly polarized light beam. A black shield prevented light from shining on the sides of the tank and being reflected from them. The irradiance of this beam on the tank was approximately 100 foot lamberts. Two opposed, matched projection lamps were placed so that their beams were parallel to the plane of polarization; a second pair was placed perpendicular to the first (Fig. 2). The brightness of these lamps could be varied with neutral density filters or by a variable transformer. Light intensities were measured with a model 501M Photovolt photometer.

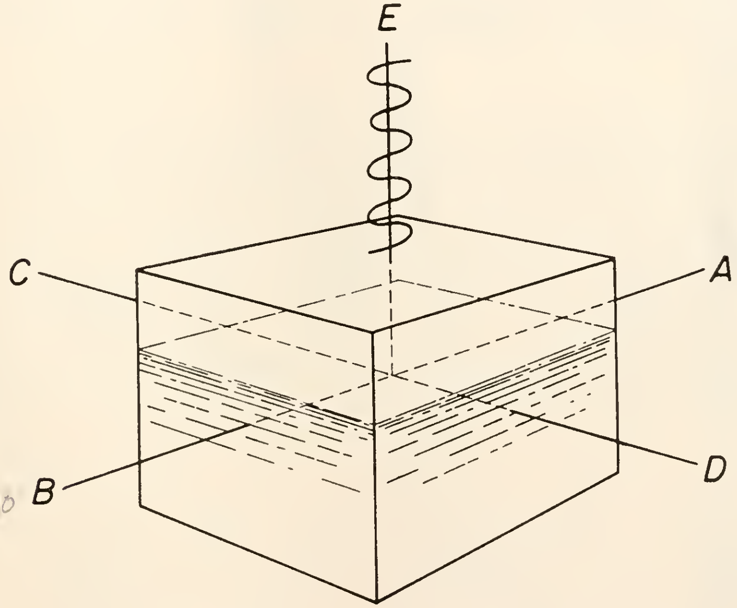

FIGURE 2. Diagram of test tank and its illumination. AB and CD are pairs of horizontally opposed light beams; E is a vertical beam polarized in ABE plane. CD is perpendicular to AB.

To record the orientation of the swimming animals during a test, a time exposure photograph of three seconds was made. The path of each moving daphnid in the field was represented by a line on the photograph. The directions of these lines were measured with a protractor, and the measurements were grouped into twelve intervals of 15° each. The midpoints of these intervals were 0° = (180°, line AB in Fig. 2), 15°, 30°, 45°, . . . 165°. The 0° azimuth was parallel to the plane of polarization of the overhead light beam (E in Fig. 2) and also to one pair of horizontally opposed light beams (AB in Fig. 2). In the experiment without the overhead polarized light two pairs of horizontally opposed light beams were employed, one at the 0° azimuth and the other at the 90° azimuth. In another set of experiments in which only side lights were used, the single pair of opposed horizontal beams was parallel to the 90° azimuth.

For convenience in discussing and manipulating the data we may calculate an

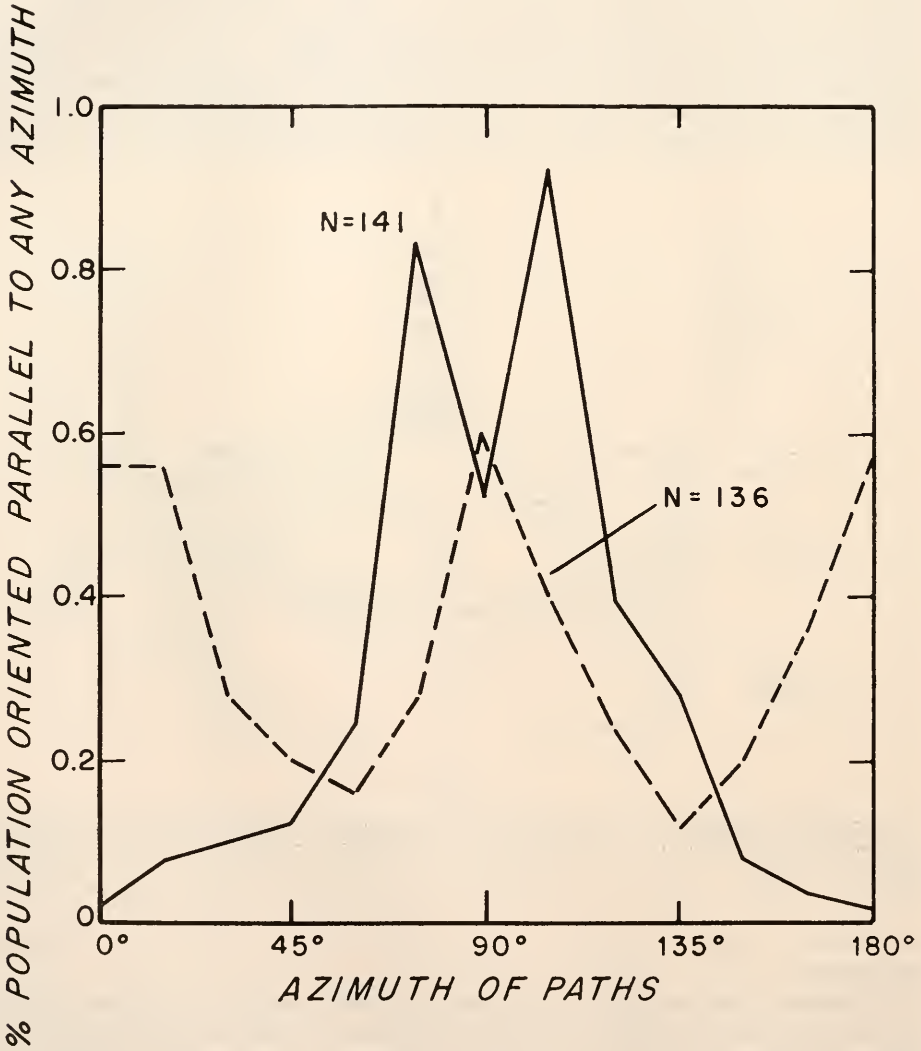

FIGURE 3. The per cent of a population of *Daphnia* oriented at various azimuths relative to horizontal, nonpolarized light beams. Solid line represents the data from a single pair of opposed light beams parallel to the 90° azimuth. Dotted line represents data from two pairs of opposed beams perpendicular to each other, one pair parallel to the 0° azimuth, the other pair parallel to the 90° azimuth.

index of the angular orientation relative to any given azimuth from the following relation:

$$\frac{P_\theta - P_{\theta+90°}}{P_\theta + P_{\theta+90°}} = \text{I. O., the index of angular orientation:}$$

where P_θ is the ratio between paths parallel to the azimuth heading θ and all paths, and $P_{\theta+90°}$ is the ratio of paths parallel to the azimuth heading $\theta + 90°$ and all paths. The measurements of orientation were grouped by 15° intervals as stated above. In presenting the data, running averages of three groups are used and thus orientation for a given angle includes all organisms oriented within 22.5° of that angle. Since each ommatidium subtends an angle of 50°, greater precision of orientation implies some integration of receptor information. The index of orientation can vary from plus one to minus one with zero being an indication of equal amounts of behavior in both directions, which would include random behavior.

RESULTS

The solid line of Figure 3 shows the orientation of 141 *Daphnia* to a single pair of horizontally opposed light beams. The response, with maxima at 75° and 105°, is approximately parallel to the light beams which are directed along the 90° azimuth. The average index of orientation at these two peaks is 0.87. The lower index of orientation at 90° is unexplained but has been reproduced in several experiments. The dotted line shows the orientation of daphnids to four matched lights 90° apart in the horizontal plane. The responses to the two perpendicular pairs of beams are parallel to the beams and are nearly equal with an index of orientation of −0.09.

Results of the experiment with one pair of opposed beams, described by the solid line of Figure 3, appear to support the assumption that daphnids possess a positive phototaxis and orient by maintaining maximum light intensity in the forward ommatidia.

The experiment with two pairs of opposed beams shows that when the front and lateral ommatidia are equally illuminated, the population of daphnids has equal numbers of animals orienting to each pair of opposing beams and thereby further supports the assumption that positive phototaxis is guided by maintenance of maximum intensity in the forward ommatidia. The data show that the daphnid compound eye does not act simply as a receptor consisting of a large number of parts, each obeying a cosine^2 law, where the intensity at the receptor will be equal to some constant times the cosine^2 of the angle of incidence. If the daphnid eye did obey the cosine^2 law, the 4-beam experiment would produce random results. That the daphnid eye could not obey the cosine^2 law is also clear because each ommatidium subtends an angle of approximately 50°, thereby limiting the angle through which each ommatidium can receive light directly.

We know from the 4-beam experiment what the response of a population of daphnids is when the front and lateral ommatidia are equally illuminated. What, then, will be the effect of an overhead polarized beam in combination with one pair of horizontally opposed lateral beams which can be varied in intensity to produce various ratios of overhead polarized light intensity to lateral nonpolarized light intensity?

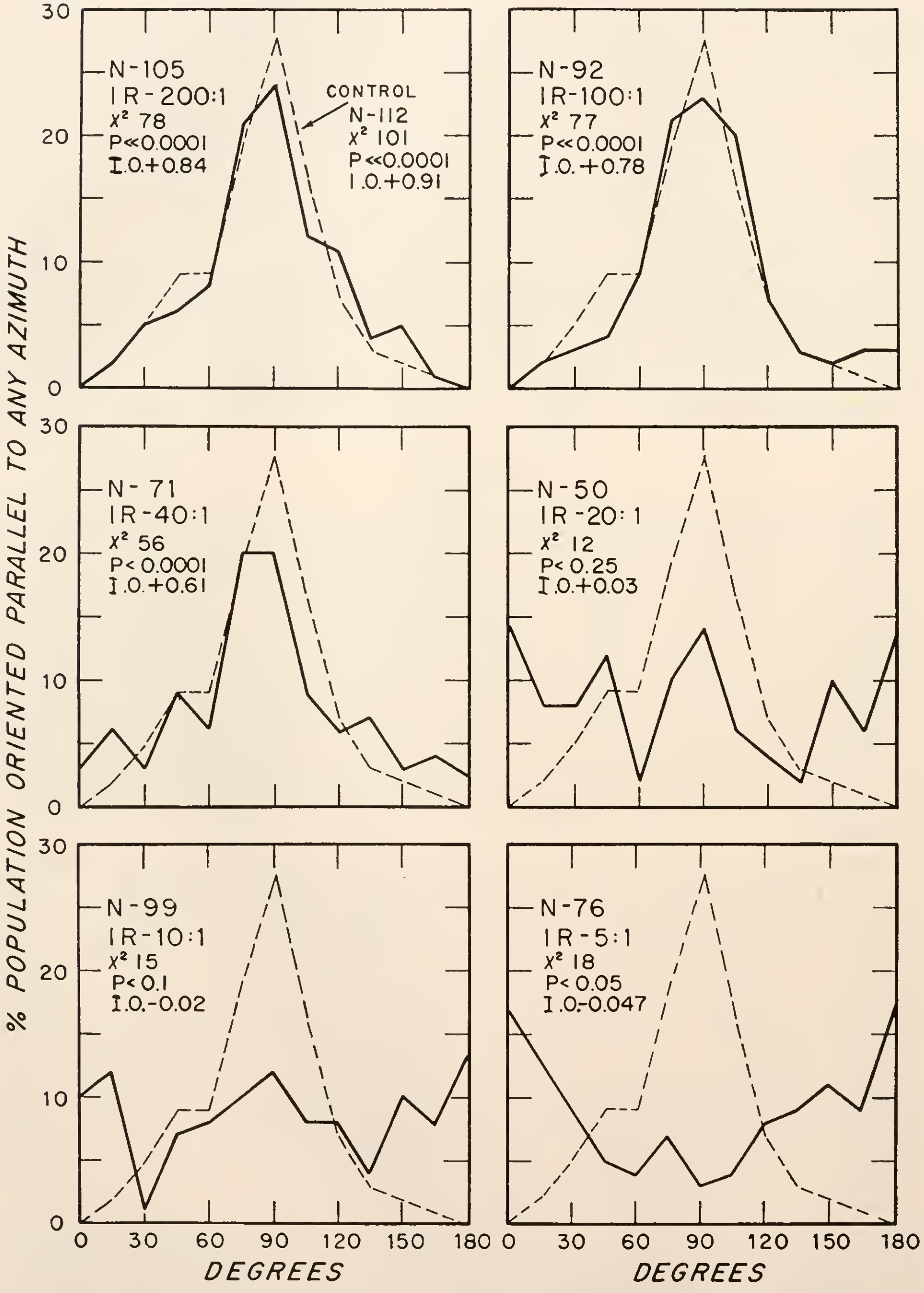

FIGURE 4. Orientation of *Daphnia* at various azimuths relative to the plane of polarization (0–180°) at six different ratios of intensities of vertical to horizontal light beams. The vertical beam is polarized and at a constant intensity. The pair of horizontal, opposed beams is parallel

TABLE I

A summary of data

Intensity ratio	Number of paths measured	Chi²	*P*	Index of orientation
Control	112	101	.0001	+0.91
200:1	105	78	.0001	+0.84
100:1	92	77	.0001	+0.78
40:1	71	56	.0001	+0.61
20:1	50	12	0.25	+0.03
10:1	99	15	0.10	−0.02
5:1	76	18	0.05	−0.047
2 Beams	136			−0.87
4 Beams	176			0.09

Figure 4 shows six graphs of the response of *Daphnia* to different intensities of lateral light, the overhead polarized beam remaining constant. The ratios of the overhead polarized intensities to side nonpolarized intensities (IR in Fig. 4) were chosen so that some were higher than the balance point ratio predicted on a theoretical optical basis, and some were lower. Each graph is plotted with the data from a control experiment, shown as a dotted line, in which only the vertical polarized beam is present. The data on the graphs are summarized in Table I. In the control, Chi square for the null hypothesis that the direction of swimming is random is 101, giving a probability much less than 1 in 10,000 that the behavior is random. The Chi square tests for the different intensities of lateral light are included in Figure 4. An examination of the graphs in sequence from that showing an intensity ratio (IR) of 200:1 to that of 5:1 shows a gradual change in orientation. At 200:1 and 100:1 the effect of lateral light intensity is minimal. At 20:1 the Chi square test gives a probability of the orientation being different from random orientation of only 0.25, showing that at this ratio the intensity apparent to the animal is nearly the same parallel and perpendicular to the plane of polarization. At the ratio of 5:1 the taxic response is oriented more toward the lateral light than it is to the stimulation offered by the polarized beam.

The calculated ratio of overhead polarized intensity to side nonpolarized intensities produced at the rhabdomeres of two perpendicular ommatidia by a vertical beam of polarized light is 8:1, as shown in Figure 1. The index of orientation with this ratio is +0.91. Throughout the range of intensity ratios of vertical to horizontal illumination there is a graded response. This graded response is best seen in Figure 5 where the index of orientation is plotted against the intensity ratios. Because the magnitude of the electroretinogram is proportional to the logarithm of the intensity of the stimulating light (Hartline, 1930), the intensity ratios of Figure 5 were plotted as logarithms. The data points seem to fall in a straight line

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to the plane of polarized light and varies in intensity. Abbreviations: N is number of animals used; IR is the intensity ratio of overhead polarized to lateral nonpolarized light beams; χ^2 is Chi square value; *P* is the probability that the orientation is random; I.O. is index of orientation.

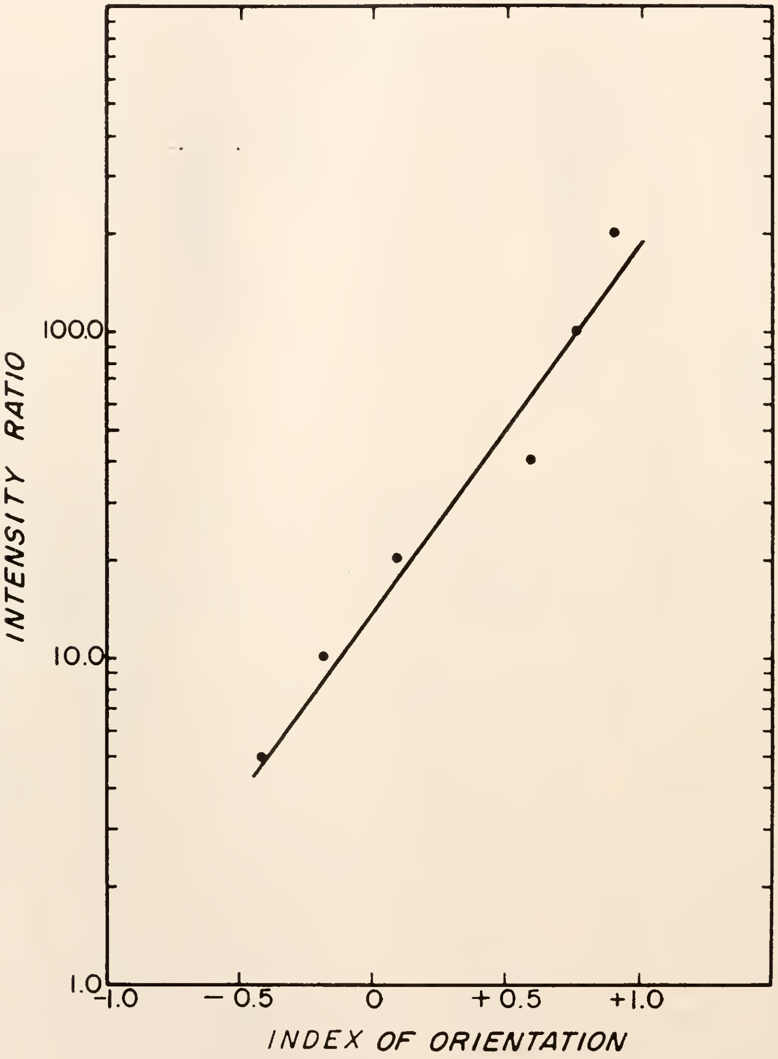

FIGURE 5. The degree of orientation at various intensity ratios of vertical to horizontal light beams. The scale of the intensity ratios is logarithmic.

which was drawn by eye. We interpret the intercept of this line with the zero value of the abscissa to be the ratio at which the intensities in the lateral and forward ommatidia are equal. From the graph this value is approximately 15:1. By theory this ratio should be approximately 8:1 as calculated for Figure 1. In view of the uncertainty of the assumptions made concerning the index of refraction and the shape of the lenses we think this discrepancy is small.

The data do not, of course, distinguish between the Brewster-Fresnel internal reflection model and all other models. A single refraction with the light path direct to visual pigments (Stephens *et al.*, 1953) would produce maximum intensity in lateral ommatidia when daphnids orient perpendicular to the polarization plane. On the other hand, a refraction followed by a reflection would produce maximum intensity in the forward ommatidia when daphnids are similarly oriented. If responses to polarized light are based on the same physiological mechanisms as positive phototaxes (and the 4-beam experiment strongly supports this hypothesis), then the Brewster-Fresnel refraction model of Stephens *et al.* is ruled out for daphnids, but the Brewster-Fresnel internal reflection model is not ruled out.

In a further attempt to test the assumption that the orientation to polarized light is essentially a phototaxic response in which the forward ommatidia are kept bright, we studied the behavior of daphnids made photonegative by drugs or by ultraviolet light. In these animals we expected to find orientation parallel to the plane of polarization rather than perpendicular, but this expected orientation did not occur. The failure of photonegative daphnids to orient parallel to the polarization plane has constituted a major criticism of the Brewster-Fresnel reflection model (personal communication from Colin Pittendrigh and from Rudolph Jander). Clearly, we must resolve this apparent paradox or abandon the model altogether.

The paradox may be resolved if two separate and distinct phototaxes are involved. The primary and obvious phototaxis response of pilocarpine-treated daphnids to an intense parallel light beam is a vigorous negative phototaxis. The secondary phototaxic response of these animals is a weakly positive phototaxis to any dim light beam perpendicular to the intense beam. This paradoxical behavior of daphnids is consistent with their possession of two separate photoreceptors having quite different functions (Baylor and Smith, 1957) : the compound eye is sensitive to polarized light, whereas the naupliar eye appears to control the sign of phototaxis and geotaxis in response to a number of chemical and physical factors of the environment. The behavior of daphnids in the natural habitat shows an obvious adaptive value for these two distinct and separate phototaxes executed approximately at right angles to each other. A negative and a positive phototaxis to the sun are presumably useful for guiding vertical migration, and at the same time a positive phototaxis for light scattered from phytoplankton or other food particles permits food-finding during the day when daphnids are photonegative. In daphnids made vigorously photonegative by treatment with 10^{-6} M pilocarpine, the change in behavior produced by adding a horizontal beam to the vertical polarized beam was compared with the same experiments in which the animals were untreated and photopositive. Results of preliminary experiments show no significant difference between vigorously photonegative drug-treated daphnids and untreated photopositive daphnids. The data points from these experiments fall on the curve of Figure 5.

We are hopeful of finding another drug which will reverse the secondarily positive phototaxis normally associated with finding food. When this is done we may then anticipate that such animals treated in this way will orient parallel to the polarization plane of an overhead light.

SUMMARY

1. Daphnids illuminated by a single vertical beam of polarized light swam approximately perpendicular to the polarization plane.

2. Daphnids illuminated by a single pair of opposed horizontal beams of light oriented toward the brighter light of the pair.

3. Daphnids illuminated by two pairs of opposed horizontal beams set at right angles to each other swam in the beam of the brighter pair of light beams.

4. Daphnids illuminated simultaneously by three beams (one polarized and coming from overhead, the other two nonpolarized and horizontally opposed, parallel to the polarization plane of the overhead light) responded to the overhead polarized light when its intensity was greater than 20 times that of the horizontal beams. When the intensity of the overhead beam of polarized light was less than 20 times that of the horizontal beams, the daphnids responded to the horizontal opposed beams instead of the polarized beam from overhead.

5. The changes in behavior induced by various intensity combinations of overhead and horizontal light beams were in good agreement with the changes predicted from daphnid eye structure.

6. Daphnids exhibiting drug-induced negative phototaxis were shown to possess simultaneously a secondary weak positive phototaxis always executed at right angles to the negative phototaxis. This weak positive phototaxis at right angles to the negative phototaxis is proposed to account for photonegative daphnids which orient perpendicular to the polarization plane of a vertical beam of light.

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KARYOPLASMIC STUDIES IN HAPLOID, ANDROGENETIC HYBRIDS OF CALIFORNIA NEWTS[1]

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The combination of a nucleus of one species acting in the cytoplasm of another is theoretically ideal for the study of the roles of the nucleus and cytoplasm in the differentiation of characters which distinguish the species. The means for achieving interspecific karyoplasmic combinations has been by heterospermic fertilization of eggs devoid of active maternal chromosomes. The preponderance of interspecific karyoplasmic hybrids in amphibians has been androgenetic haploids (Fankhauser, 1955; Moore, 1955). Although repeatedly attempted in the past, these haploids typically die prior to the appearance of recognizable species characters. Of 21 interspecific androgenetic, haploid hybrid combinations enumerated by Fankhauser (1955), none developed to a stage permitting an analysis of the relative influence of the foreign haploid nucleus or the cytoplasm on a specific character.

In a classic experiment, Hadorn (1936) overcame the difficulty of rearing haploid hybrids by grafting haploid tissue of *Triton palmatus* cytoplasm and *T. cristatus* nucleus to diploid homospermic *T. alpestris* hosts. The postmetamorphic skin of *palmatus* is characterized by projections formed by flattened epidermal cells; the skin of *cristatus* is smooth. The grafted haploid hybrid skin on metamorphosed *alpestris* hosts possessed projections typical of *palmatus,* the cytoplasmic donor in the hybrid merogon. This species character, although it appears late in development, has been considered to be "determined" in the egg cytoplasm prior to insemination, *i.e.,* the character is an expression of the genotype of the diploid oocyte from which the egg was derived. As was recognized by Hadorn, a complicating factor in this experiment is that the epidermis of *alpestris,* the diploid host, also forms skin protuberances.

Dalton (1946) produced hybrid merogons of *Taricha* (*Triturus*) *rivularis* cytoplasm and *T. torosa* nucleus. The two species differ strikingly in larval pigment patterns. Dalton transplanted haploid hybrid merogonic neural crest to diploid *torosa* hosts. The transplanted haploid hybrid tissue produced a pigment pattern essentially like that of *torosa,* the nuclear contributor. However, an early influence of the cytoplasmic donor, *rivularis,* was manifested in the rate of melanization and distribution of the pigment cells.

The circumvention of the early demise of haploid hybrid tissue by transplantation to diploid embryos has been of value, but in order to rule out the possibility of any

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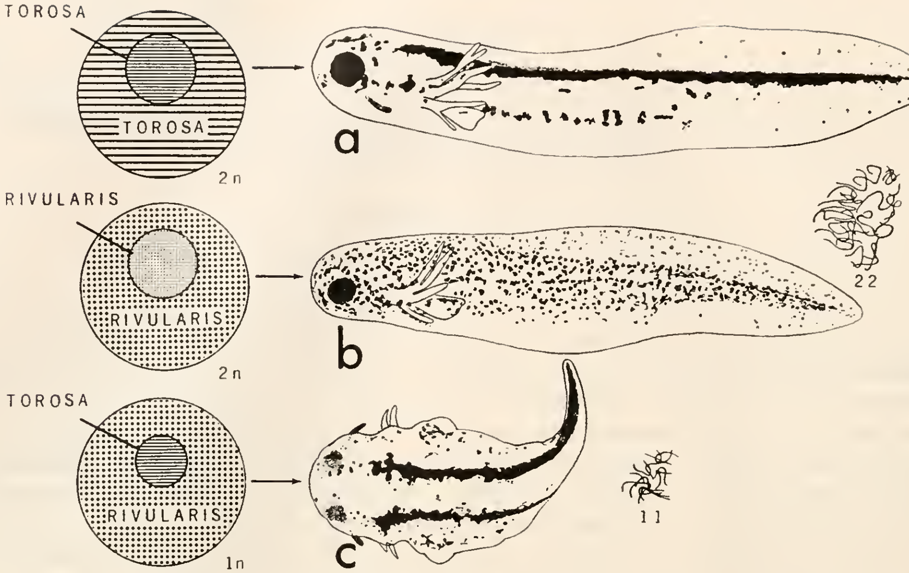


FIGURE 1. Drawings of larval pigment patterns and balancers: a, homospermic diploid *T. torosa* (paternal nuclear contributor); b, homospermic diploid *T. rivularis* (maternal cytoplasmic contributor); c, androgenetic, haploid hybrid. Schematic karyoplasmic constitutions depicted on the left; camera lucida drawings of diploid (22) and haploid (11) chromosome complements on the right.

influence of the host tissues on the differentiation of species characters, it is still desirable to obtain whole haploid hybrid larvae. During the course of an experiment designed to study gene-dosage effects in polyploid hybrids (Brandom, 1960), two of 54 larvae from *Taricha rivularis* eggs fertilized with *T. torosa* sperm were haploids. Both developed to stages where distinctive species characters were readily visible. The parent species are advantageous for karyoplasmic studies by virtue of their distinctive larval pigment patterns and the formation of viable diploid hybrids. The larval melanophores of *rivularis* are distributed over the lateral and dorsal body surfaces (Fig. 1b), whereas those of *torosa* are confined primarily to compact dorsal bands (Fig. 1a). The diploid hybrid is intermediate to the parent species. Another character, the balancer, is always fully developed in *torosa,* but rudimentary or absent in *rivularis.*

The results of a few selected experiments bearing on the analysis of the localization of factors which direct the differentiation of species characters are summarized in Table I. The conflicting results of these experiments stress the need for further experimentation that might aid in the clarification of the karyoplasmic problem. The present report deals with this problem.

MATERIALS AND METHODS

The methods employed have been described in detail elsewhere (Brandom. 1960). Eggs of *Taricha rivularis* were fertilized by sperm of *T. torosa,* heat-shocked at 35° to 37° C. for ten minutes, and returned to room temperature. Those

Table I

The role of the nucleus and the cytoplasm in the determination of specific characters

Organisms	Authors	Localization of the factors for the determination of specific characters	
		Nucleus	Cytoplasm
Haploid, androgenetic hybrids			
Sea urchins	Boveri, 1889	+*	
	Hörstadius, 1936	+	
	von Ubisch, 1953	+	
Amphibians	Hadorn, 1936		+
	Dalton, 1946	+	+**
	Sambuichi, 1952	+	
	Humphrey and Fankhauser, 1957	+	
Diploid, androgenetic and nuclear-transplant hybrids			
Insects	Astaurov and Ostriakova-Varshaver, 1957	+	
Amphibians	Sambuichi, 1957	+	
	McKinnell, 1960	+	
	Gurdon, 1961	+	

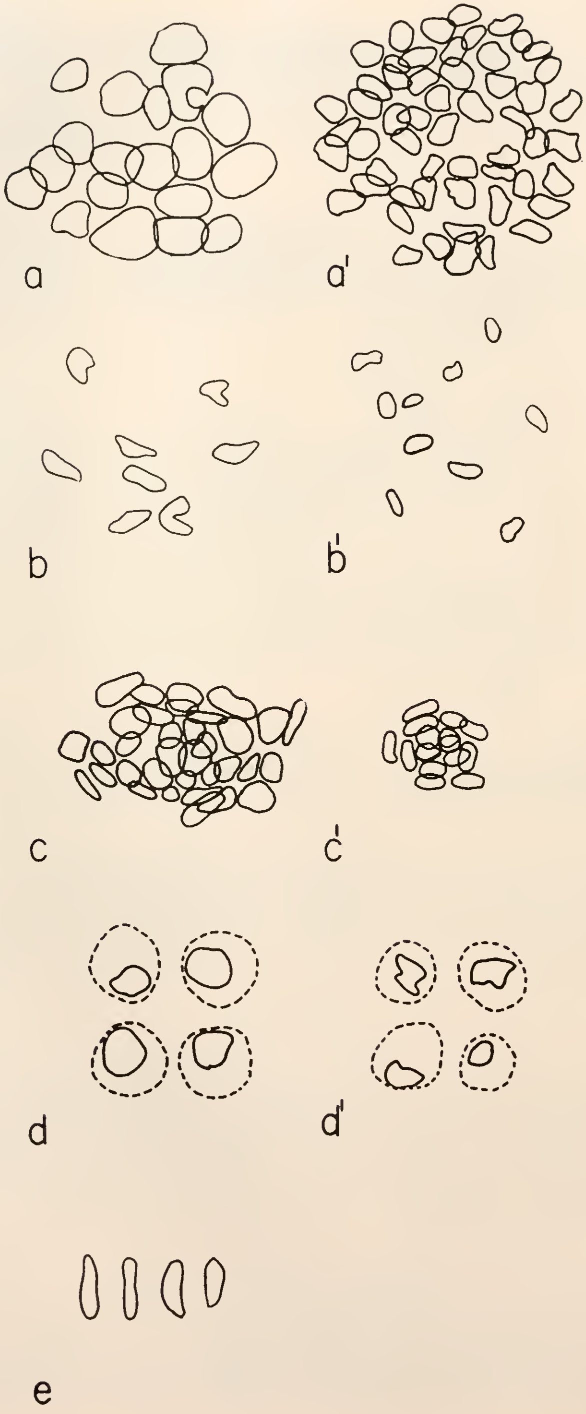
* Of historical interest; results later re-interpreted (Boveri, 1918).
** Partial, early cytoplasmic effect.

embryos which survived to larval stages were tail-clipped, fixed in Bouin's fluid, the tail-tips stained in Mayer's acid haemalum and mounted whole. In addition to direct chromosome counts in the tail-tips, heteroploidy was confirmed by the comparison of nuclear and cell size in whole-mount tail-tips and microsections.

RESULTS

Heat-treated *rivularis* eggs (4,369) fertilized with *torosa* sperm yielded two haploid, androgenetic hybrids which developed to mid-larval stages (Twitty stage 39; see Rugh, 1962). Although exceptional homospermic haploids have been reared to more advanced stages (Baltzer, 1922; Fankhauser, 1937, 1938), these are the first haploid, androgenetic hybrid amphibians to develop to a stage where species characters are discernible.

The controls for this experiment were larvae of the two homospermic diploid species and diploid *rivularis/torosa* hybrids. In addition, homospermic haploid *torosa* were obtained in heat-shock experiments conducted prior to the hybrid experiments. The haploid homospermic larvae have a normal *torosa* pigment pattern (see Dalton, 1946, p. 195). No homospermic heat-treated *rivularis* eggs have developed into haploids (Brandom, 1960). However, it may be assumed,


a
a'
b
b'
c
c'
d
d'
e

based on other homospermic haploid experiments, that the pigment pattern of haploid *rivularis* larvae would not be qualitatively altered.

The two haploids did not differ noticeably from control larvae in cleavage rates. Marked developmental difficulties were first noted in yolk-plug and neurula stages. Large yolk-plugs persisted up to early tail-bud stages, and the neural folds closed irregularly. Yolk extrusion was observed through wounds in the ventral body wall of both haploid larvae. In early tail-bud larvae pronounced edema in the heart, gill, and forelimb-bud regions remained until the time when the embryos either died or were fixed. Alleviation of fluid pressure by surgical means did not materially reduce the edemic condition. The fluid imbalance and dwarf appearance of our haploids are two of the characteristics normally associated with the haploid amphibian syndrome. Microcephaly occurred in one haploid, but in the other the head was near-normal when the animal was fixed. No early localized breakdown in head mesenchyme, a difficulty previously noted in some haploid hybrids of European *Triton* (Baltzer, 1930), was found in our material.

1. *Tissue and organ architecture*

One haploid hybrid ceased development after the appearance of larval species characters but deteriorated before it could be fixed for sectioning; the other was fixed in good condition. The nuclei and cells of the haploid hybrid were smaller and more numerous than those in comparable areas in the diploid controls. This is illustrated by the outline drawings of tissues in the tailtips (Fig. 2) and microsections (Fig. 3). Limited nuclear pyknosis was observed in the brain but the haploid central nervous system contained mostly normal cells. The notochord was bi- and tripartite in some regions; anteriorly it was single, posteriorly it became progressively divided by thickening partitions into two and then three divisions. Duplication of the notochord was previously reported in homospermic haploid *torosa* larvae (Dalton, 1940).

The kidney tubules in the haploid were more numerous and contained larger lumina than those of the diploid control. It is not known whether there is a functional relationship between the abnormalities of the kidneys and the fluid imbalance. Rafferty (1961) concludes from homoplastic transplantation experiments (haploid to diploid and diploid to haploid kidney transplants) that factors other than the haploid kidney are likely to be involved in the fluid imbalance syndrome.

The shape of cells in the lens of the eye and the nephric duct is more cuboidal than comparable cells in the diploid control (Fig. 3). The tendency of haploids to approximate normal organ and body size in the face of decreased nuclear and cell size is partially achieved by a compensatory adjustment in cell shape and cell number (Fankhauser, 1955). As might be expected on the basis of observations of homospermic haploids, the architecture of the heterospermic haploid cells is subordinated to the achievement of near-normal organ size.

FIGURE 2. Drawings of nuclei from larval tailtips of diploid and haploid hybrids of *T. rivularis* ♀ × *T. torosa* ♂: a–e, diploid; a′–e′, haploid. Reading from top to bottom: epidermal interphase nuclei; mesenchyme cell nuclei; nuclei of lateral-line organs; epidermal glands (dotted outline) and nuclei; red blood cell nuclei (absent in haploids) × 540.

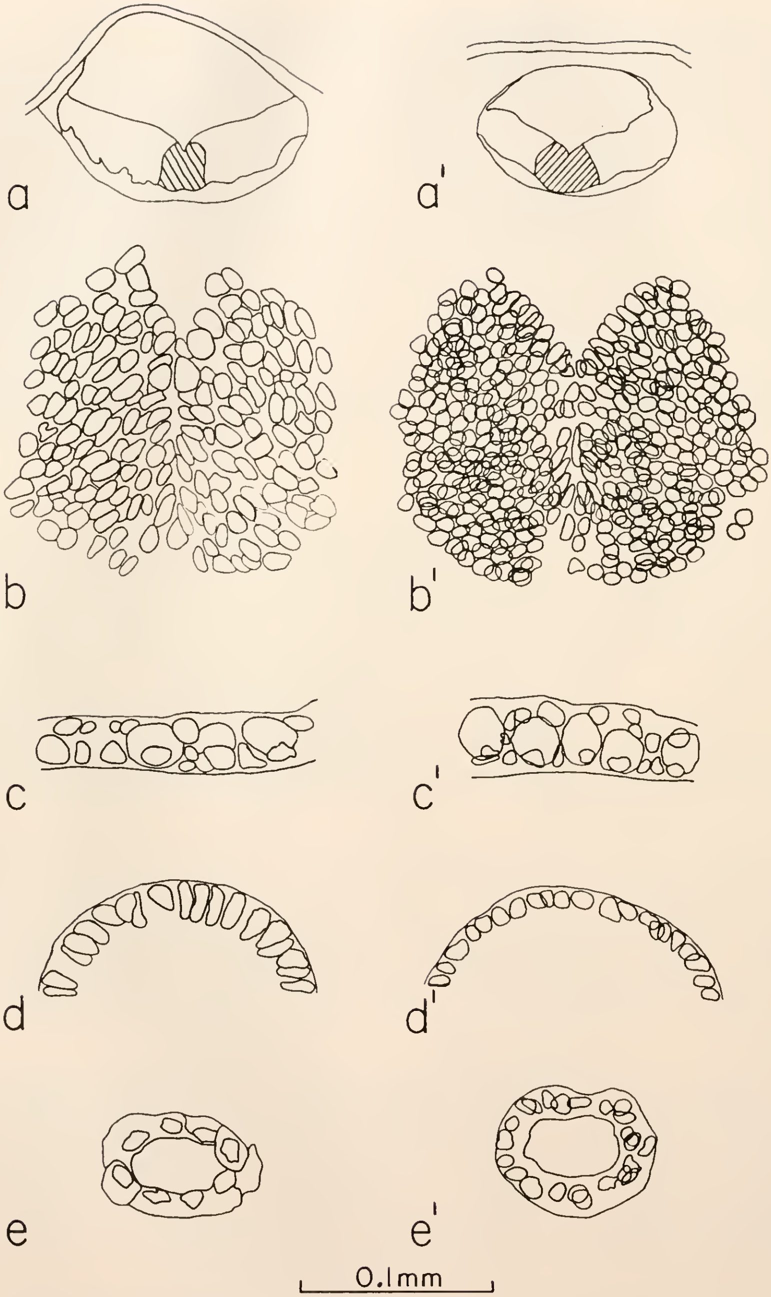


FIGURE 3. Projection-drawings of microsections of diploid (a–e) and haploid (a′–e′) hybrids of *T. rivularis* ♀ × *T. torosa* ♂: a, a′, low-power drawings of myelencephalon; b, b′, nuclei from shaded areas of a and a′; c, c′, cells and nuclei of glands of the epidermis; d, d′, nuclei of peripheral layer of the lens; e, e′, nephric ducts.

2. *Balancer*

As is characteristic for mountain stream-dwelling salamander larvae, the balancer is either rudimentary or absent in *rivularis,* whereas in *torosa* this organ is fully developed (Twitty, 1936). The balancer is always present in the diploid hybrid of *rivularis* ♀ × *torosa* ♂, although it may be reduced in comparison with homospermic diploid larval *torosa.* In the heterospermic haploids, the balancer was fully developed (Fig. 1). Thus, the *torosa* nucleus acting in *rivularis* cytoplasm directed the development of this organ into a strictly nuclear-donor structure.

3. *Larval pigment pattern*

The banded arrangement of the larval melanophores of the haploids was dominantly like that of the paternal nuclear contributor, *torosa* (Fig. 1). A few melanophores were visible on the flanks, but they were not in excess of those found in homospermic *torosa* larvae. Although a slight effect of the *rivularis* cytoplasm cannot be ruled out as a possibility, the random arrangement of the few melanophores on the yolk area can be ascribed to physical disturbances of the larval pigment pattern as a consequence of the extreme ventral and lateral body swelling. In support of the latter alternative, no strong evidence of *rivularis*-like early pigmentation was observed.

Discussion

Although the present report is concerned with nuclear-cytoplasmic haploid hybrids, several experiments involving diploid nuclei of one species acting in the cytoplasm of another bear on the problem of the differentiation of species characters.

Astaurov and Ostriakova-Varshaver (1957) reported the first adult karyoplasmic hybrids. Diploid, androgenetic hybrids of *Bombyx mandarina* and *B. mori* were obtained by temperature shock and x-ray treatment to fertilized eggs. The parent species differ in distinctive morphological characters. *Mandarina* caterpillars are of dark markings while *mori* caterpillars have different markings depending on the race. *Mandarina* moths are dark greyish-brown, while those of *mori* are white or cream-colored. In the *mandarina* cytoplasm plus *mori* nucleus combination, the species characters were all like those of *mori.* The cytoplasm did not visibly affect the species characters of the hybrid. None of the hybrids of *mori* cytoplasm plus *mandarina* nucleus developed to imagos, but four individuals were typically nuclear-like in body size, larval markings, and other characters.

Employing the nuclear transplantation technique of Briggs and King (1953), Sambuichi (1957) transplanted diploid nuclei of *Rana nigromaculata brevipoda* into enucleated eggs of *R. n. nigromaculata.* Larval character differences in these two subspecies include tadpole color, labial tooth formula, and shape of the tail. The young metamorphosed frogs differ in dorsal and ventral color pattern. The diploid hybrids are intermediate to the parents in all the characters. With one exception, the embryos, tadpoles, and young frogs of *nigromaculata* cytoplasm plus *brevipoda* diploid nucleus contained only characters of the nuclear-donor subspecies. The exceptional individual later became *brevipoda*-like.

McKinnell (1960) transplanted nuclei of kandiyohi dominant-mutant *Rana pipiens* into wild-type *Rana pipiens* egg-cytoplasm. Three of the intraspecific karyoplasmic chimeric tadpoles underwent metamorphosis and each had pigment patterns similar to the nuclear donor, kandiyohi.

Gurdon (1961) transplanted nuclei between two subspecies of *Xenopus laevis* (*X. l. laevis* and *X. l. victorianus*). The two subspecies differ in the time of appearance of the larval body and anal melanophores and in postmetamorphic color and color patterns. The nuclear transplant larvae and frogs all showed the distinguishing characteristics of the subspecies which provided the nucleus.

Returning to the haploid experiments, Boveri (1889) first attempted combining the nucleus of one species with the cytoplasm of another by fertilizing egg fragments of *Sphaerechinus granularis* with sperm of *Parechinus microtuberaculatus*. Boveri's pioneer work on sea urchins was criticized on several counts by Morgan (1895) and Seeliger (1896) and, upon repeating his earlier experiments, he showed that it was not possible to produce viable haploid hybrid merogons that would develop beyond gastrulation (Boveri, 1918). The limited development of whole haploid, androgenetic hybrid sea urchin embryos was partially overcome by Hörstadius (1936). He surgically combined the presumptive skeletal micromeres of haploid *Paracentrotus* ♀ × *Psammechinus* ♂ hybrid with homospermic ectodermal and endodermal cells of *Paracentrotus*. In these germ-layer chimeras, the larval skeleton resembled the species which furnished the nucleus of the skeletal cells. More recently, von Ubisch (1953) obtained good merogonic hybrid plutei of *Sphaerechinus* cytoplasm plus *Psammechinus* (or *Paracentrotus*) nucleus. Skeletal characteristics and ciliated bands of the merogons all showed characters of the species which contributed the nucleus.

Finally, Humphrey and Fankhauser (1957) produced intraspecific haploid hybrids between wild, dark (DD) and recessive, white (dd) axolotls by cold-shock treatment of fertilized eggs. The embryos were predominantly white haploids, the recessive color of the males and therefore of androgenetic origin. Only one dark haploid was obtained, presumably of gynogenetic origin.

Concerning temperature shock as a means of inducing androgenesis, Böök (1945) has proposed that cold shock, if it affects the egg when it is in the second anaphase, may cause a paralysis of the spindle. According to this hypothesis, the egg chromosomes remain in the anaphase without being able to reorganize a resting nucleus. A return to normal temperature activates the sperm nucleus; the egg nucleus anaphase configuration does not have the same attraction for the sperm nucleus as does the metaphase, and the result is that the centrosome of the sperm nucleus divides, resulting in a haploid embryo with paternal chromosomes. The mode of elimination of the maternal chromosomes in the androgenetic hybrids reported herein is not known. However, since both of our haploids were androgenetic and all of Humphrey and Fankhauser's (1957) axolotls were androgenetic except one, some such mechanism may be operating in the great majority of haploids derived from temperature-shocked amphibian eggs.

The architecture of the heterospermic haploid tissues was the same as was observed in homospermic haploids (Fankhauser, 1955). Compared to the diploid hybrid controls, the cells of the heterospermic haploids are greater in number but smaller in volume (Figs. 2 and 3). Adjustment in cell shape in single-layered tissues and organs in order to maintain near-normal organ size also agrees with prior observations on homospermic haploids (Fankhauser, 1945).

The embryological basis for specific larval pigment patterns has been extensively investigated by extirpation, transplantation, and tissue culture experiments (Twitty, 1945, 1949; Twitty and Niu, 1948, 1954). Both *in situ* and when explanted in coelomic fluid, *torosa* melanophores migrate out, become highly melanized, and then secondarily reaggregate. Under the same conditions *rivularis* melanophores neither differentiate as fully nor reaggregate as strongly as do those of *torosa*. The two species also differ in the number of larval chromatophores; *rivularis* melanophores are more numerous than *torosa*. These and other findings of Twitty and his co-workers permit us to consider the qualitative changes in the larval pigment patterns in the haploid hybrids as due to quantitative, gene-mediated changes in the pigment cells themselves.

A genetic effect of the single *torosa* genome acting in *rivularis* cytoplasm was discernible in the number of larval melanophores. Although difficult to quantitate because of the secondary banding, there were fewer melanophores in the haploid hybrids than in the diploid *rivularis/torosa* larvae. This suggests that the nuclear-donor species (*torosa*) is exercising a strong action that tends to override a typical consequence of haploidy. Ordinarily, the number of larval pigment cells is greater in homospermic haploids than in homospermic diploids (Fankhauser and Schott, 1952).

The melanophores in the two haploid hybrids were densely pigmented like those of homospermic, diploid *torosa* larvae. Hence, a diminishing effect on the melanization of the larval melanophores by the *rivularis* cytoplasm was not seen. Interpreted in the light of Twitty's findings, the higher grade of differentiation of the haploid pigment cells (visibly manifested by their highly melanized state) qualitativly affected the pigment pattern. The aggregation into dense dorsal bands in homospermic *torosa* is due to the retraction of intercellular processes and occurs only with the attainment of advanced melanophore differentiation characteristic for this species (Fig. 1a). The larval pigment pattern of the haploid, androgenetic hybrids indicates that the *torosa* nucleus was the locus of the factors which determined this larval species character (Fig. 1c).

The fully developed balancer in the heterospermic haploid (which is absent or rudimentary in *rivularis*) emphasizes the strong directive influence of the *torosa* nucleus in the progressive acquisition of this species character.

The lack of species characters of the cytoplasmic donor, *rivularis*, does not exclude the possibility that the cytoplasm produced profound but unseen effects on the propigment and balancer cells before stages when these cells were well differentiated, and subsequently assumed the larval pigment pattern and balancer characteristic for the nuclear-donor species. These results do show that the cytoplasm does not materially affect the specific characters of whole haploid *rivularis/torosa* hybrids during those stages when the visual recognition of species characters can be made.

The author is grateful to Dr. Victor C. Twitty for direction, support, and encouragement during this investigation. Thanks are also due Dr. Gerhard Fankhauser for appraising the manuscript in its incipient form.

SUMMARY

Two species of West Coast newts differ strikingly in larval pigment patterns. *Taricha torosa* has a banded arrangement of the larval melanophores; in *T. rivularis* the larval melanophores are dispersed. *Torosa* is also characterized by a well developed balancer, whereas in *rivularis* the balancer is either absent or rudimentary.

1. Two of 54 heat-shocked, interspecific hybrids of *T. rivularis* ♀ × *T. torosa* ♂ were haploids. The two haploids are the first amphibian androgenetic, haploid hybrids to develop to stages where species characters could be observed.

2. The tissue and organ architecture of the heterospermic haploids conform to prior findings in homospermic haploids. The nuclei and cells are smaller and more numerous than in the diploid controls. A compensatory adjustment in cell shape as well as cell number was observed in single-cell layered organs.

3. The balancer was fully developed in the heterospermic haploids, thus indicating a strong directive influence of the nucleus (*torosa*) in the formation of this organ.

4. The larval pigmentation was dominantly like the nuclear-donor species in the number, degree of melanization, and pattern formation of the melanophores. No evidence was found of an influence on pigmentation by the cytoplasmic-donor species.

5. The above findings are discussed in relation to other studies on the roles of the nucleus and the cytoplasm in the differentiation of species characters.

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RESPONSES OF THE PLANARIAN, DUGESIA, AND THE PROTOZOAN, PARAMECIUM, TO VERY WEAK HORIZONTAL MAGNETIC FIELDS[1]

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The question of whether living things are sensitive to terrestrial magnetism has undoubtedly fleeted through the minds of innumerable persons since this geophysical factor first became known. But neither the naturalist, observing the behavior of organisms in the field during their continuing responses to the myriads of more obvious physical factors, nor the experimental biologist, casually testing the response of living things to artificial magnetic fields, even very strong ones, found any consistent evidence that living creatures perceived this weak terrestrial force. It is a common observation that animals in nature may come to bear at any given moment apparently all possible compass relations in their bodily orientation; orientation of the normal resting or foraging animal to the horizontal component of magnetic field would be expected generally to be of no adaptive consequence.

In recent years, however, two kinds of phenomena have come to the forefront in biological research which are exceedingly difficult, in some instances impossible, to account for in any orthodox physiological terms. These two phenomena are the apparent persistence, in rigorously controlled constancy of all the more obvious factors of the physical environment, of biological senses of time and space in their terrestrial relationships. The first includes the mechanism for timing the well-known persistent daily, tidal, monthly, and annual periodisms. The second involves the still completely mysterious "map sense" or capacity to localize position in space which is so evident in a wide spectrum of kinds of animals capable of "homing."

Also demanding a rational explanation is the means by which organisms, even when screened from fluctuations of every obvious weather-related factor, still display variations in metabolic rate, even of quite substantial magnitudes, correlated with the essentially aperiodic weather disturbances (Brown, 1959, 1960). There remains no reasonable doubt that organisms are sensitive to some subtle geophysical factors which pervade the ordinary "controlled" conditions of the physiology laboratory. Fluctuations in these unidentified factors must bear information related to weather changes, as well as to terrestrial time and space.

In a series of recent experiments, it has been demonstrated that the marine mud-snail, *Nassarius obsoletus,* is able to perceive small changes in strength of the horizontal component, H, of a magnetic field close in strength to that of the earth's natural field (Brown, Brett, Bennett and Barnwell, 1960; Brown, Webb and Brett, 1960; Barnwell and Webb, 1961). The snail can, furthermore, distinguish between directions of the fields, both the earth's and weak ones produced by bar magnets

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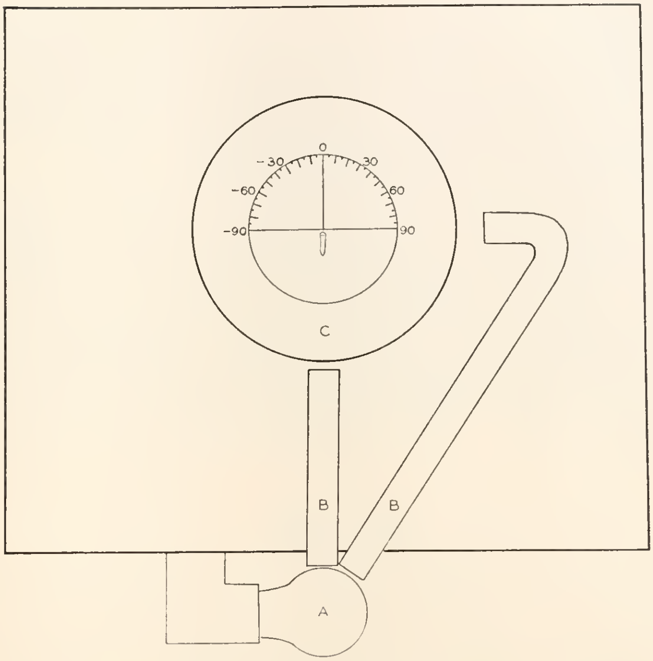

FIGURE 1. The apparatus employed in the study of response of *Dugesia* to magnetism, showing arrangement of (A) 7$\frac{1}{2}$-watt opalescent lamp; (B) sleeved light-conducting glass tubes; (C) Petri dish centered over a polar coordinate grid.

(Brown, Bennett and Webb, 1960; Brown and Webb, 1960; Brown, 1960; and Brown and Barnwell, 1961). The character of the response to magnetic field exhibits rhythmic changes that are regulated by the solar-day and lunar-day "clocks" of the snails, and by a synodic monthly one. The following study was conducted to learn whether such responsiveness was of wider biological distribution.

METHODS AND MATERIALS

The common, small flatworm, *Dugesia dorotocephala,* was selected as an animal possessing convenient behavioral characteristics for study, and simultaneously being non-marine and phylogenetically very distantly related to the snails. The worms were collected twice a year, in early October and in June, from a small spring-fed stream in Fox River Grove, Illinois. They were maintained in darkened containers in the laboratory, and fed liver twice weekly.

The apparatus consisted of a 3$\frac{3}{4}$-inch glass Petri dish centered over a polar coordinate, paper grid (Fig. 1). This apparatus was set inside a black-lined wooden cabinet; use of ferromagnetic materials was carefully avoided in the whole assembly. The apparatus was continuously illuminated from a 0.5-inch circular source about 16 inches directly above the center of the grid, provided with a 7$\frac{1}{2}$-watt opalescent lamp which yielded an illumination of about five lux at the Petri-dish level. A second small light provided a weak horizontal source of illumination parallel to the zero axis of the grid, and a third, horizontal weak light source provided illumination parallel with the 90° axis, from the right side. The horizontal light sources were onion-skin paper-covered ends of black-sleeved, 10-mm. solid glass rods leading light into the cabinet from a $7\frac{1}{2}$-watt opalescent lamp attached rigidly to the outside of the cabinet. To minimize other, uncontrolled illumination sources, the experiments were conducted in a darkened room.

In operation, a planarian was quickly oriented with a fine brush toward the zero axis of the polar grid, just short of its origin, as illustrated in Figure 1. The deviation in worm path from the initial direction was then recorded in terms of the point, to the nearest 5°, at which the worm crossed the circular arc one inch from the origin. Measurement of the times to reach the one-inch line was found for 22 paths to average 15.45 seconds; standard deviation was ±2.02 seconds. There were no obvious orienting cues in the system other than the two horizontal light sources. The single vertical light source provided no cue. Any unintentional bias due to asymmetry of any other factors, such as extraneous reflected illumination, remained essentially unchanged throughout the period of experimentation.

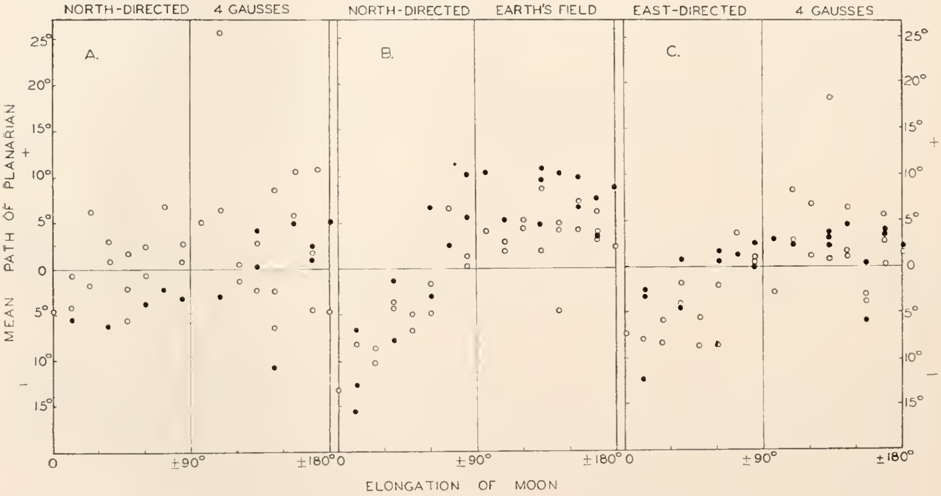

FIGURE 2. The mean angular path of initially N-directed planarians as a function of elongation of the moon (0° = New Moon; 180° = Full Moon); with (A) a North-oriented magnetic field of 4 gauss, (B) the earth's field alone and (C) an experimental field of 4 gauss, with South pole directed Eastward. Solid circles, first experimental series (November and December); open circles, repetition of experiment (January).

Beneath the apparatus were calibrated slots into which an 18-centimeter Alnico bar magnet could be placed horizontally at distances to produce any one of a series of horizontal field strengths, with the south pole rotatable to any desired compass direction to supplement, subtract from, or otherwise modify, the natural horizontal field.

It should be recalled at this time that by convention of physicists, the earth's northern pole is a magnetic S pole and the southern pole, a magnetic N one.

ORIENTATION OF NORTH-BOUND WORMS IN A SINGLE, VERTICAL-LIGHT FIELD

During about a two-month period from November 8, 1960, through January 2, 1961, 4677 planarian paths were recorded,[2] 2172 in the earth's field alone and 1766 in a four-gauss field, with the S pole directed East. The apparatus was at all times directed magnetic north. A single horizontal light, that one parallel to the zero axis of the grid, was used. It was left on only until the moment the worm reached the origin. It was then extinguished. The observations were made on about 24 occasions, never more than one series on any given day, distributed over the whole period. In very few cases, with too small sample sizes on a given day, the data were combined with those of the succeeding day. The observations were always made sometime between 9 A.M. and 3 P.M., to minimize any daily rhythmic change that might occur, comparable to that previously demonstrated for the snails. For each sample series 45 to 140 paths were recorded for each in the earth's field alone and in the E-W four-gauss experimental field.[3] On six occasions over the period November 25 through December 3, and on seven occasions from December 19 through January 2, each observational series included the recording of paths under three conditions. These were in the aforementioned two fields, and now, in addition, in a four-gauss field with the S pole directed north. This last field augmented the earth's natural 0.17-gauss north-directed horizontal one. The total number of paths observed in the N-S field was 739.

TABLE I

Planarian paths

Date	No magnet			E. W. magnet			N. S. magnet		
	N	Mean	σ	N	Mean	σ	N	Mean	σ
Nov. 25–6	82	+10.00	38.2	85	+2.60	25.0	85	− 3.24	35.1
Nov. 28	83	+ 9.50	32.2	59	+2.55	23.1	53	+ 4.06	39.9
Nov. 29	51	+10.20	29.6	46	+4.80	26.5	55	−10.45	43.7
Nov. 30	98	+ 6.42	36.2	77	+0.58	25.6	89	+ 5.17	39.4
Dec. 1–2	140	+ 8.03	33.4	72	+2.43	19.9	77	+ 5.20	47.0
Dec. 3	57	+ 5.80	36.6	47	+3.51	22.4	54	+ 2.32	35.7
	σ Range 29.6 − 38.2° M = +8.25 ± 1.53° σ = 34.6 ± 1.08°			σ Range 19.9 − 26.5° M = +2.68 ± 1.22° σ = 24.0 ± 0.87°			σ Range 35.1 − 47.0° M = −0.85 ± 2.00° σ = 40.6 ± 1.42°		

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[2] The author wishes to acknowledge his indebtedness to the several persons, especially to Young H. Park, Bertil S. Thunstrom, and Andrew Bertagnolli, who devoted many hours to acquiring the data of this study.

[3] The stated horizontal field strengths in this report are the fields present at the level of the worms when the bar magnet is oriented to oppose the earth's own horizontal vector and differs from these in the expected manner as it is rotated to other directions in the earth's 0.17-gauss field. The values are accurate only to about ±10% as a consequence of pole-strength variations among the individual magnets employed. The field strengths were initially computed, but later were confirmed with a Rawson gaussmeter.

A second and more systematic study, involving 3493 worm-paths, 1186 in the earth's field, 1181 in the N-S, and 1126 in the E-W magnetic fields, was conducted between December 28, 1960, and January 31, 1961. There were 29 observational series, involving 29 different days; each series included observations under each of the three conditions of experimental fields.

The mean path of the worms in solely the earth's field exhibited throughout the three-month period a synodic monthly fluctuation, the worms turning maximally counterclockwise at the time of new moon and maximally clockwise over the fortnight centered on full moon. In Figure 2B, the solid circles indicate the mean path in degrees as a function of elongation of the moon (angle between sun-earth and moon-earth axes) for the worms in the earth's field for the first experimental study, and the open circles for the repetition of the experiment, or second study. In Figure 2C are the mean paths at the same times as measured for the four-gauss E-W experimental field, and in Figure 2A for the N-S experimental field. In every single daily series in the first set of experiments, when the worms were turning clockwise in the earth's field, the experimental E-W field effected less clockwise turning, and when the worms were turning counterclockwise, the E-W field effected less counterclockwise turning. This is illustrated, in part, in Table I for the relatively stable values obtained during the full-moon semi-month, November 25–December 3.

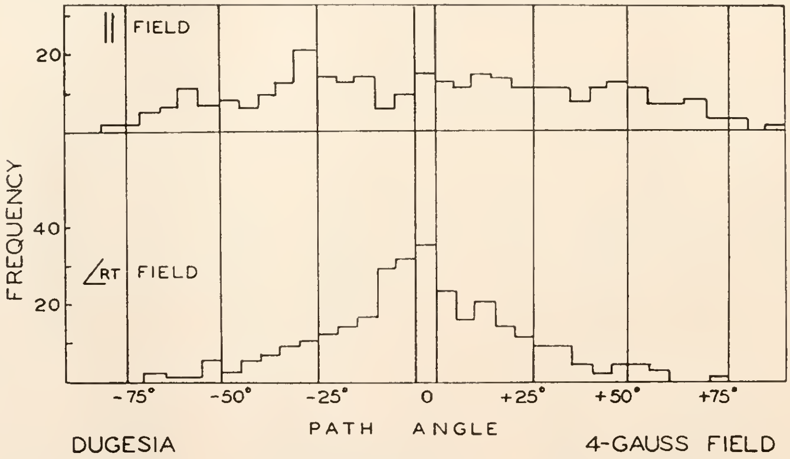

FIGURE 3. Frequency distributions of planarian paths in the 4-gauss N-S field (upper) and E-W field (lower), during the period November 25–December 3, 1960 (see Table I), a period during a late autumn full-moon semi-month.

The worms were apparently able to distinguish between the E-W and N-S four-gauss fields on the one hand, and between either of these and earth's field on the other, as is evident both from inspection of Figure 2 and from the statistical analyses shown in Table I. In Table I, it is seen that the mean paths of the worms in the E-W and N-S fields were not significantly different from one another, but the standard deviations were clearly so. The increased standard deviation in the N-S field over the E-W one reflected a conspicuous increase in turning, but now both clockwise and counterclockwise. The effect is evident from comparison of the two frequency distributions in Figure 3.

The planarian orientational response in the earth's field alone appeared, during this study, to be a function of elongation of the moon, over nearly three 360° cycles. The relationship seemed to be roughly symmetrical about 0°. The limits of clockwise and counterclockwise response appeared, within the error of measurement, to be phase-synchronized with the upper and lower lunar transits.

From Figure 2, it is evident without need of recourse to statistical analysis that the monthly cycle, so obvious in the earth's magnetic field alone, was partially suppressed by the 4-gauss E-W field, and abolished by the 4-gauss N-S one.

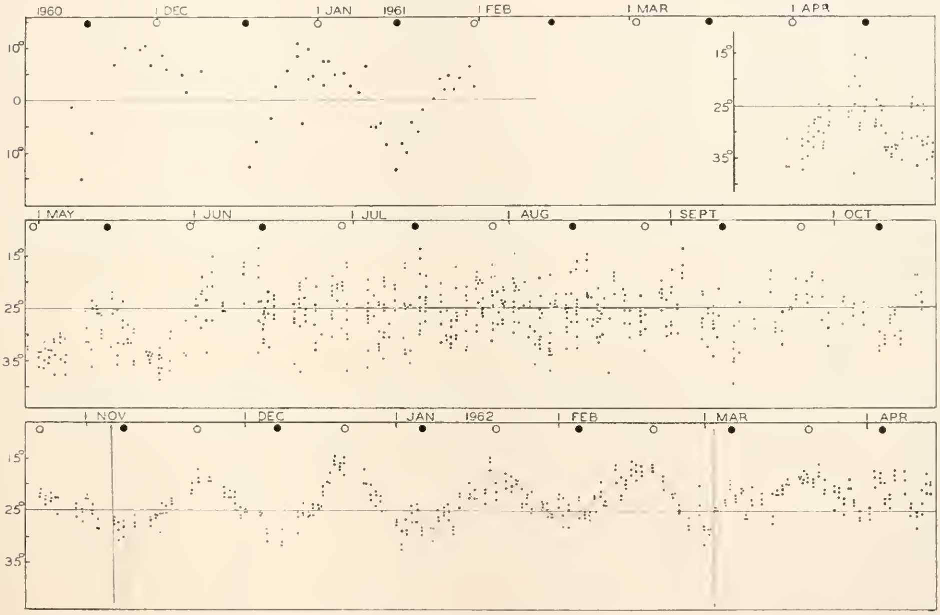

FIGURE 4. Variations in the character of the monthly orientation rhythm of *Dugesia* over about a 17-month period. From November through January, 1960, the worms were in a single vertical light field; each point was the average of 45 to 140 paths. For the remainder of the time, the worms were in a three-light field with each point the average of 15 paths.

MONTHLY RHYTHM IN NORTH-BOUND WORMS

The monthly variation in the path of the North-directed planarians during late morning and early afternoon hours was followed for more than an additional year. Data were obtained from late March through August by sorting out the numerous control samples of North-bound worms in the earth's field alone from experiments involving responses of *Dugesia* to modified fields. The latter included (1) changes in compass direction in the earth's field, (2) rotation of a horizontal, experimental 10-gauss field while animals remained continuously magnetic-North directed, and (3) differentiation of magnetic axes of horizontal fields as related to horizontal field-strength. These latter results have been reported earlier (Brown, 1962a) in a preliminary account, but will be described in more detail below.

For all these later experiments from April, 1960, onward, the illumination was different from that of the initial experiments. The worm's orientation was observed in a steady three-light field; the three lights were at 90° to one another in the arrangement described earlier.

The mean path of the worms always involved counterclockwise turning in response to the weak light on the right. This illumination pattern was adopted because the variance of paths was found to be less. Each sample now comprised always the mean of only 15 worm paths.

In Figure 4 are shown the mean paths in degrees plotted against day of the year and phase of moon from November 10, 1960, through to April 14, 1962. The clear monthly cycle, with maximum counterclockwise turning on the day of new moon and maximum clockwise turning about the time of full moon, is again evident from November 10 through January 31. Here each point is the mean of 45 to 140 worm paths. No data including mean paths of worms North-bound in the earth's field alone were obtained between January 31 and March 31.

North-bound worms in the earth's field alone exhibited a monthly variation between March 31 and about the middle of May, but now the mean path of the worms, in the three-light field, was steadily to the left and the turning relationship to lunar phase was the mirror image of the earlier observations during the preceding late fall and winter. Scatter of the mean paths was substantially greater than during the preceding period of study.

By early June, there was a suggestion of a tendency for a maximum in clockwise turning to occur near the times of both new and full moon. By the latter part of June, the scatter of the mean paths had become even greater and continued so for three or four months. There was suggestion from inspection of the data, however, of a tendency for counterclockwise turning to be greater over the quarters of the moon than over the times of new and full moon. A quantitative analysis of the paths from June 15 to August 31 proved those for the seven-day periods centered on the moon's quarters to be -26.48 ± 0.384 ($N = 157$) and those for seven-day periods centered on new and full moon to be -24.21 ± 0.393 ($N = 136$). The difference between these two was highly significant ($t = 4.1$). In other words, there appeared to be a low amplitude semi-monthly fluctuation during the summer with maximum clockwise turning at both new and full moon.

By late August, there was an abrupt inversion in lunar relationship to yield a maximum in counterclockwise turning at full moon, and another near new moon early in September. There was thereafter a gradual return to a clear monthly fluctuation with maximum left turning at new moon and right turning at full moon. The monthly fluctuation became progressively more sharply defined, with scatter of mean paths reduced, between September and November. By the latter time, the overall form and phase relations of the monthly variation had become, and remained through the winter, qualitatively like those which obtained for the corresponding months of the preceding year.

By early March, there was an abrupt alteration in phase to give a maximum in clockwise turning at new moon. This inversion, which seems to have been anticipated during the preceding two months, judging from the gradual decreasingly

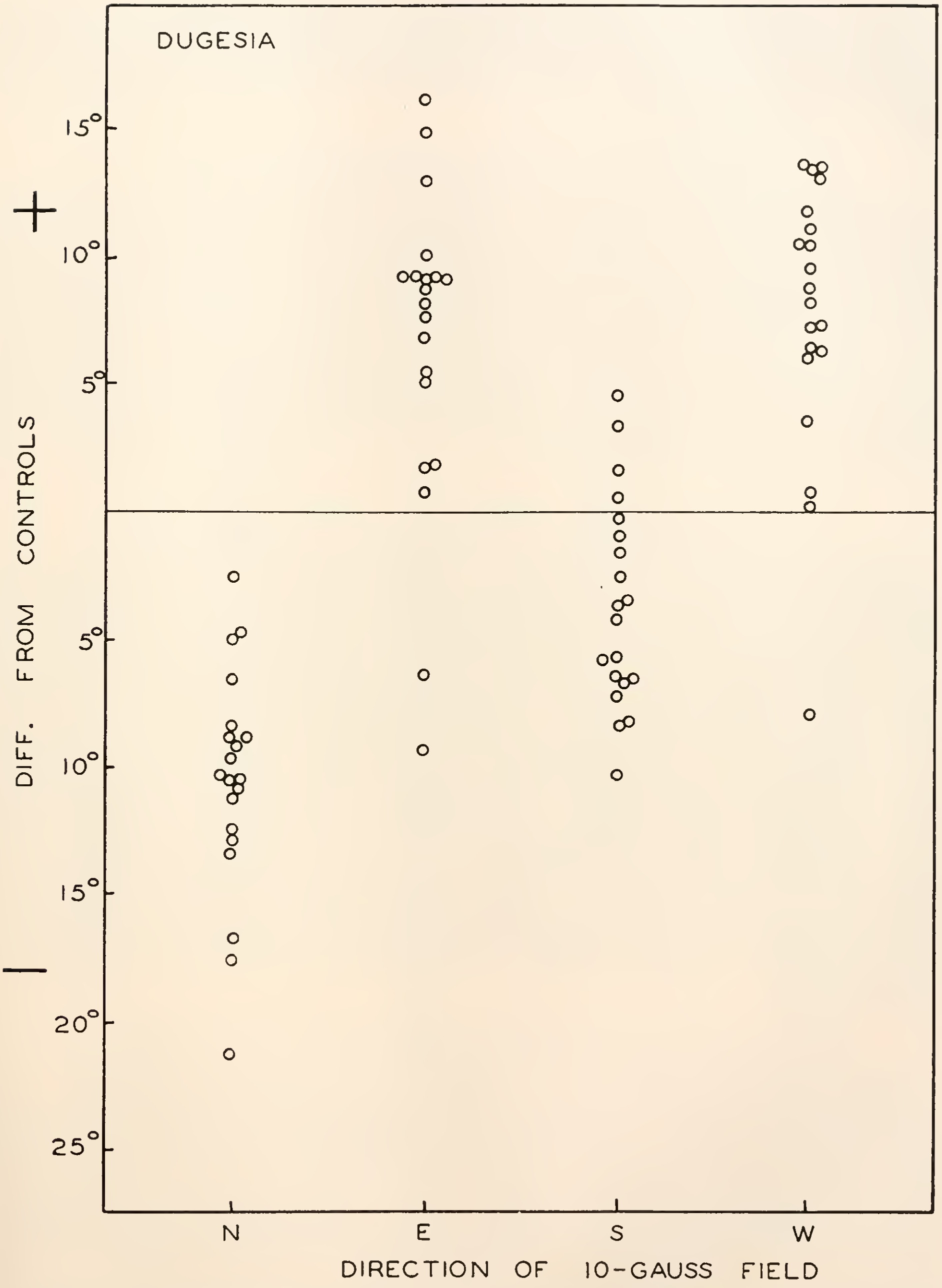

FIGURE 5. Difference between the mean paths of *Dugesia,* initially directed Northward in the earth's field, and the comparable paths when experimental, horizontal 10-gauss fields, with South-pole directed in each of four compass directions, are superimposed. Path angle is expressed as difference from interpolated controls in the earth's field alone.

strong left turning at new moon, suggested the initiation of a mirror-imaging of a monthly cycle during April to June, comparable to that observed the corresponding months of the preceding year. The variations in form of the monthly rhythm clearly suggest an annual component.

Orientation to a Ten-Gauss Horizontal Field

Between March 31 and May 5 the response of *Dugesia* to a 10-gauss horizontal field was determined as the field was rotated by 90° intervals while the orientation apparatus itself remained steadily directed magnetic North. On 20 mornings series were run consisting of each of four directions of the experimental field, with a "control" in the earth's field alone following each 10-gauss exposure. The effects of the 10-gauss field, with S-pole directed N, E, S, and W, expressed as differences from the average path of the four controls in the same-day series, is illustrated in Figure 5. The worms clearly differentiated between parallel and right-angle fields, and between N- and S-directed fields.

A Compass-Direction Effect in Earth's Field Alone

Another experiment with *Dugesia* was very informative. This was performed during the period from June 15 through September 12. It involved simply rotating the orientation apparatus by 90° intervals in a darkened room, in the earth's field alone. In this experiment, performed on 30 different afternoons distributed over the three-month period, each series included all four compass directions, in shuffled order, followed at once (in every instance but one) by a repeat of the four, again shuffled. When average path for each compass-direction was computed as the difference from the mean for the four directions of that particular group of four, the results shown in Figure 6 were obtained. The worms in the earth's field alone clearly distinguished between N-S and E-W orientations. However, the results obtained in the earth's horizontal field of 0.17 gauss and illustrated in Figure 6 are essentially the mirror-image of those depicted in Figure 5, which are the results from the rotation of the 10-gauss field. The significance of this difference was clarified from the results of the following experiment that was run concurrently.

Relation of Horizontal Field-Strength to Orientation

This experimental series was run on 24 mornings distributed uniformly over the three summer months. In this one, in shuffled order but always starting and ending with a control, and with a third control midway in the series, were experimental horizontal magnetic fields of four strengths—0.25, 2.0, 5.0, and 10.0 gauss, with South pole directed North and West. Figure 7 illustrates the results obtained by taking the difference between N and W. It is clear that between the three weaker fields on the one hand and the 10-gauss one on the other, there is a reversal in relative left-turning influence. The difference between response to N-directed and W-directed fields increases with a positive sign up to some limit, as the experimental field increasingly overrides the earth's, but then abruptly adopts a negative sign somewhere between 5 and 10 gauss.

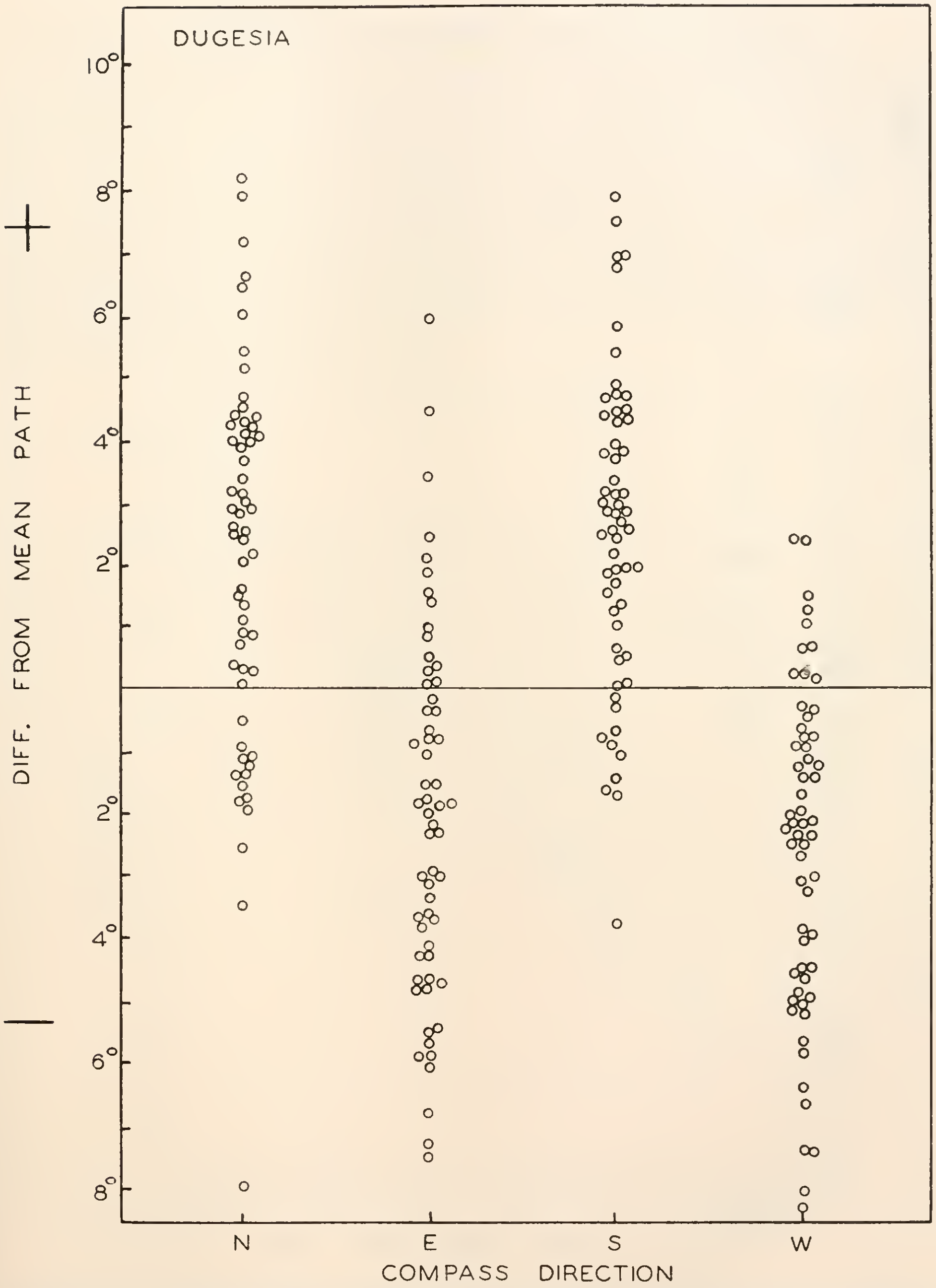

Figure 6. Difference between mean path of planarians for each four compass directions and the mean path in the same series for all four directions taken together, as the orientation apparatus is rotated to each of the four compass directions, in shuffled order, in the earth's field alone.

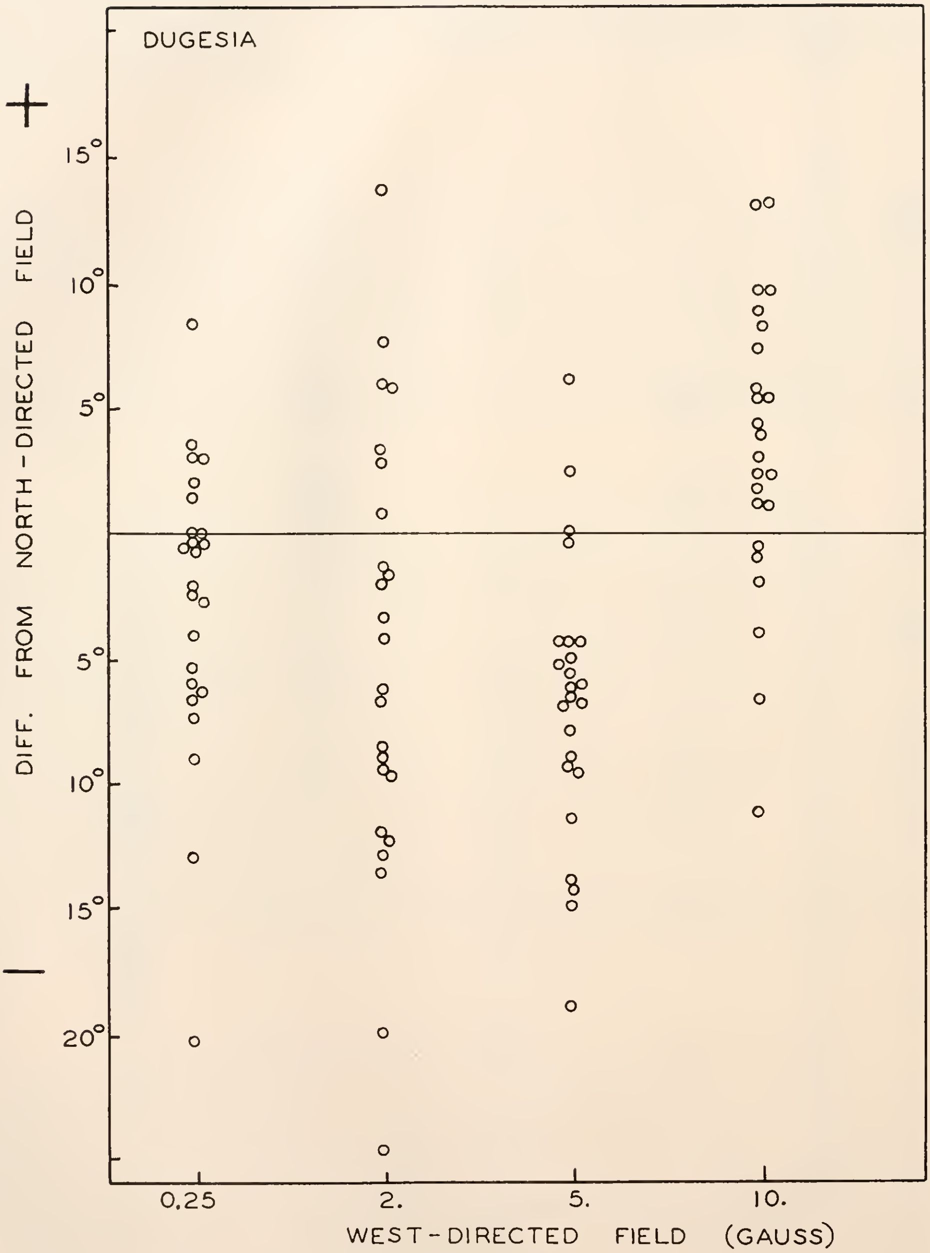

Figure 7. Difference between the mean paths of *Dugesia* initially directed Northward in the earth's field and simultaneously subjected to experimental N-directed fields to supplement the earth's to yield the values indicated, and the mean path resulting from rotation of the supplementing magnet 90° in a counterclockwise direction.

Resolution of Magnetic Field Direction

One final experimental study was conducted with *Dugesia* between June 20 and August 16, 1961. Two observers were involved, working concurrently. The experiment was performed on 21 different mornings distributed over the two-month period. For each daily series 15 worms were observed moving compass-North under each of 11 conditions presented in shuffled order. The observers were wholly uninformed of the conditions which obtained at the time of their observations. The eleven conditions included seven in which a 5-gauss horizontal field was presented at each of seven orientations at 15° intervals from S-pole directed North to S-pole directed West, and four in which the magnet was removed and the worms moved North in the earth's field alone.

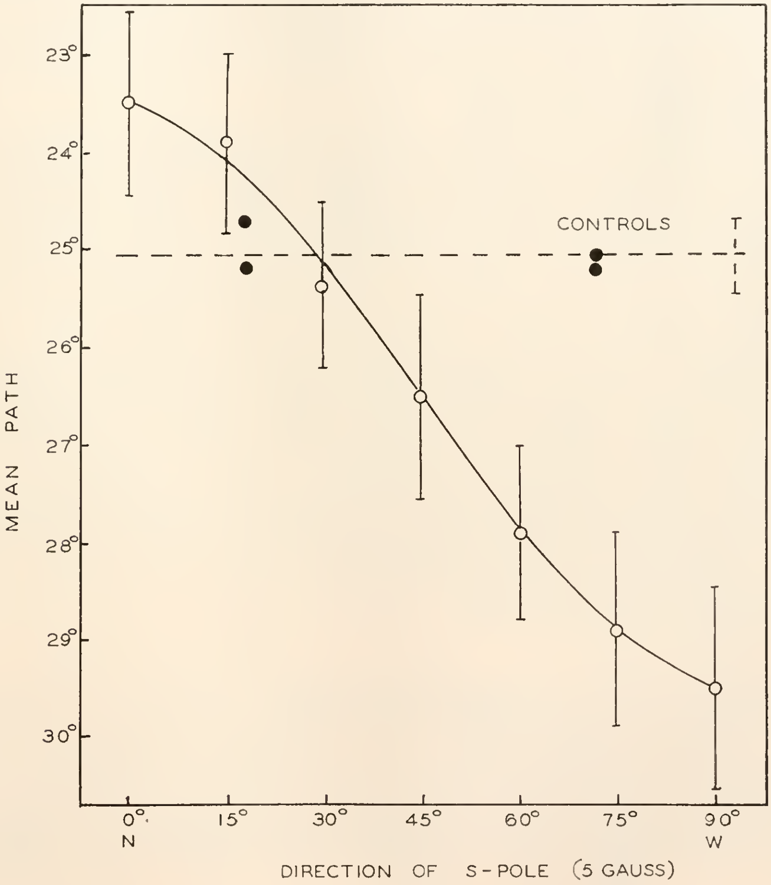

FIGURE 8. Open circles illustrate the relationship of mean path to magnet orientation for magnetic-N-directed *Dugesia* in a three-light field and subjected to an experimental 5-gauss field with S-pole changed by 15° intervals from North to West. The means for each of four successive controls in the series, for the earth's field alone, which were interpolated in random order in each experimental series, are indicated by the solid circles. Standard errors of the means are shown.

TABLE II

Path deviations from controls on same day

Angle	M	S.E.	N	Variance
0°	+1.50°	±0.97	42	34.4°
15°	+1.14°	±0.76	42	23.0°
30°	−0.36°	±0.65	42	17.1°
45°	−1.48°	±0.77	42	23.6°
60°	−2.63°	±0.83	42	26.4°
75°	−3.84°	±0.78	42	24.6°
90°	−4.46°	±0.85	42	27.4°

The mean paths of experimentals and controls for the two-month period are plotted against the conditions, in Figure 8, together with standard errors of the means. It is evident that the mean path of the worms was a function of the angle of the experimental horizontal field.

The standard errors are relatively large, in some measure a consequence of systematic fluctuations in paths of all worms, both controls and experimentals, from day to day. These latter fluctuations included a highly significant semi-monthly component. Consequently, it was not surprising to find, as shown in Table II, that when the mean paths of the experimentals were treated as deviations from the mean path for the four controls in the same series, significantly smaller errors were observed.

Two other facts were notable. As shown by Table III, the variance of the 42 mean paths in an experimental magnetic field was in every instance substantially greater than for any one of the four controls. The presence of a 5-gauss field significantly ($P < .005$) increased variance over that of controls. And whether one deals with variances of the actual mean paths (Table III) or variances of deviations from control paths (Table II), minimum variance is observed in this experiment when the worms tend to move in a path most nearly parallel with the 5-gauss horizontal field. The differences in Table II between the variances at 30° and 0° are statistically significant as determined by the test ($P < 0.01$), as is also that between 30° and 90° ($P < .05$).

TABLE III

Variances and mean paths

With magnet			Controls	
Orientation	Variance	Mean path	Variance	Mean path
0°	36.20°	23.5°	I 19.08°	24.7°
15°	34.46°	23.9°		
30°	31.05°	25.4°	II 26.80°	25.1°
45°	44.40°	26.5°		
60°	32.82°	27.9°	III 22.57°	25.2°
75	40.06°	28.9°		
90°	44.20°	29.5°	IV 25.95°	25.1°

Response of Paramecium to a 1.3-Gauss Field

It was of interest to learn whether a single-celled form exhibited such orientational responses. *Paramecium caudatum* was permitted to escape from the exit of a magnetic-South-directed, minute, funnel-shaped, aluminum corral set in the center of a 3¾-inch round Petri dish containing water 2 mm. deep. The corral exit was carefully entered over the origin of a polar coordinate paper grid (Fig. 9A). The grid was, in turn, set on the platform of a stereoscopic microscope and illuminated weakly from below by a 7½-watt incandescent lamp with opalescent glass. Between the lamp and the microscope platform was a water filter for heat absorption, and an opaque screen with a circular opening carefully centered under the corral exit. The whole apparatus was placed in a darkened enclosure. With this arrangement, the emerging paramecia were clearly silhouetted for observation.

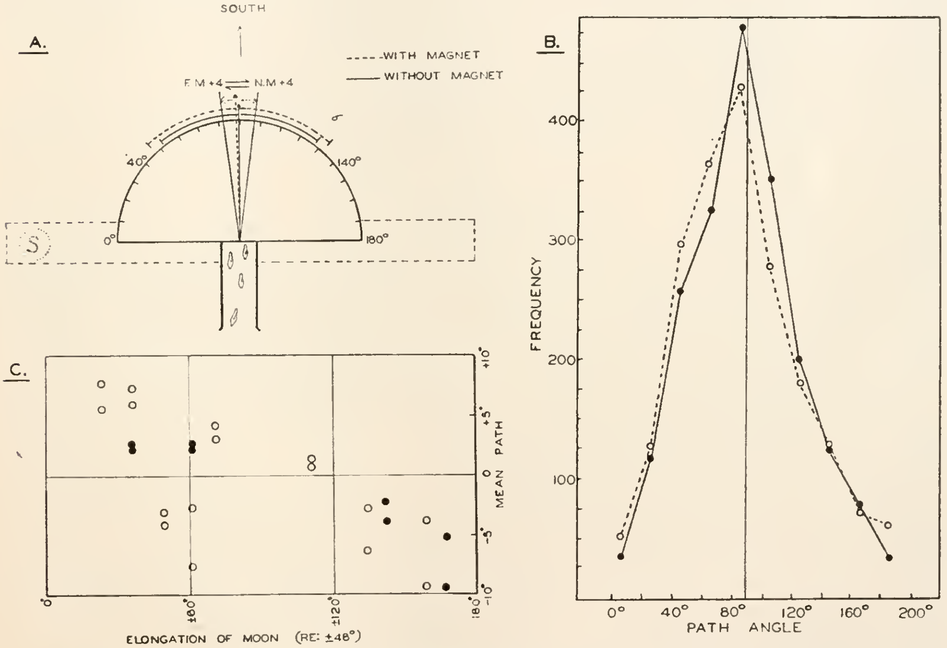

FIGURE 9. (A) Orientation of the apparatus and of the experimental bar magnet for the *Paramecium* study, illustrating mean paths in the natural and experimental field, the range of mean paths in the apparent monthly cycle, and the standard deviations of the paths. (B) The distribution of paths of *Paramecium* in the earth's magnetic field alone (solid line) is compared with the distribution when the experimental 1.3-gauss, E-directed field is superimposed (broken line). For purposes of this illustrated comparison, the values for the controls were increased proportionately to make the total number equal to that of the experimental series. (C) The relationship between mean path and elongation of moon treated as deviations from the fourth day after new moon.

Each experiment consisted of alternating observations of (1) a few paramecia making their exit in the earth's magnetic field alone with (2) a few fully comparable exits when the magnetic field was altered by an 18-centimeter Alnico bar magnet, placed horizontally and centered directly below the exit with S-pole pointed East.

The distance of the magnet was such that the strength of the horizontal East-directed component was 1.3 gauss. This horizontal strength is about eight times the earth's H component; the total field is only two to three times the earth's total field, F. Although the microscope base was predominantly constructed of non-ferromagnetic materials, two pairs of steel screws symmetrically placed two to three inches east and west were present and the microscope arm was of ferrous metal. However, that the earth's field and experimental 1.3-gauss field were not significantly distorted was assured by placing a small compass in the place of the corral. The angle of deviation of the paths from the initial southward course was determined by the point at which the animal crossed a circle perimeter with 0.5-inch radius, centered at the origin of the grid.

Between the dates February 14 and March 9, 1961, a total of 3774 individual paths were observed, 1762 in the earth's field and 2012 in the experimental, magnetic field. These data were obtained exclusively between the hours 8:30 A.M. and 4:30 P.M., but chiefly between 2:30 and 4:30 P.M. The observations were made on 12 different days during the 24-day period of study.

There was a strong positive correlation between the mean paths of the samples run in the experimental magnetic field and the control samples on the same day ($r = 0.86 \pm 0.09$). There was also a highly significant difference between the variances of paths under the two magnetic conditions. The stronger, imposed East-West field produced a highly significantly greater amount of deviation of paths from South, both clockwise and counterclockwise. Expressed as standard deviation of paths, a value of $37.7 \pm 0.638°$ was found for the earth's field and $41.3 \pm 0.656°$ for the stronger, E-W oriented one. There was, therefore, clearly an influence, highly statistically significant, of the field-strength change, whether one ascertained probabilities by the F or t test ($F = 1.20$; $t = 3.93$).

Analysis of the data indicated that there was no significant difference between the mean path of those paramecia in the 0.17-gauss South-directed earth's field and of those in the East-directed 1.3-gauss field.

However, the comparative distributions of frequencies of paths for paramecia in the 1.3-gauss E-W field and in the earth's South-directed field alone are shown in Figure 9B. Using a Chi-square test to measure the probability that the two samples were drawn from the same population, a value of $\chi^2 = 29.95$ with 9 degrees of freedom was obtained ($P < .001$). Inspection of the figure suggests this highly significant difference to be a consequence in large measure of an overall shift of the crown of the distribution curve for the animals in the experimental field to the left of that of the controls in the earth's alone.

In view of the previously demonstrated synodic monthly fluctuation in mean path of both mud-snails and planarians, the *Paramecium* data were examined for the possible existence of a comparable periodism. Inspection of the mean paths as a function of time revealed a distinct suggestion that the paramecia, too, displayed a monthly fluctuation. The inspection suggested that maximum clockwise turning was occurring for paramecia about four days after new moon (Feb. 19) and maximum counterclockwise turning about four days after full moon (March 6). In fact, computed correlations, with elongation of the moon considered as an intrinsic time series, corroborated this suggestion. With elongation of the moon expressed as ±180-degree deviation from four days after new moon (+48.8°) the value of the coefficient of correlation, r, was 0.76 ± 0.09, $N = 24$. This was higher than that found in any other phase relationship with respect to the natural monthly cycle (Fig. 9C).

Since only a 24-day period (ca. 290°) was involved in the study, it is obviously not possible to conclude with great confidence that the period of this long-cycle fluctuation in mean path was, indeed, a monthly one. However, that it probably was a monthly one is suggested since extremes of both clockwise and counterclockwise response appeared to occur within the 24-day period, and the interval between the estimated maximum clockwise and the estimated maximum counterclockwise turning seemed clearly consistent with it being 180°. Indirect support for such a cycle is considered to come from the now far better established occurrence of this period in comparable orientations of the two other previously investigated species.

Just as for the snail and planarian, one very conspicuous influence of magnetic field is upon the turning tendency in the field, without respect to whether it is clockwise or counterclockwise. It seems probable that the character of response of paramecia to an increase in magnetic field will be found, as in the other two kinds of animals, to be functions of (1) times of lunar and solar days, and their interference derivative, the synodic month, and (2) the direction of the H-component of magnetism with relation to the long axis of the body.

Discussion

Several considerations were involved in planning the present investigation. First, to be of significance under natural conditions, the organism must exhibit responsiveness to field strengths of the order of magnitude of the earth's. Any perceptive system of this sensitivity could well be expected to display little or no resolving power for fields differing greatly in strength from the earth's. Therefore, only weak fields were investigated. Secondly, to be maximally adaptive the organism would be expected to be able to differentiate the compass direction of these very weak fields. Thirdly, any response obtained might be expected to vary in a "clock-regulated" manner. And lastly, to account for a number of still unexplained biological phenomena, the responses must be postulated capable of sign reversal. For this study, the orientation of whole organisms was considered to constitute the most sensitive method for assaying any possible biological resolution of magnetic field strength and direction.

To reduce the problem to its simplest form we attempted to learn the nature of orientational tendencies or pressures in samples of a population subjected initially to enforced orientation in a highly restricted unit of space, to horizontal magnetic fields, both natural and experimental. As we have seen, the general method consisted in inducing, or permitting, organisms initially to travel in an arbitrarily decided magnetic compass direction in the earth's natural field and in experimentally altered magnetic fields, and assaying the amount the animal's paths have deviated, clockwise or counterclockwise, from the initial path after an arbitrary constant short distance was traversed.

In this present study the experimental fields that were used were only those obtained in the number 2 position of a straight bar magnet, in order that maximum simplicity could be achieved. By this means it was possible to alter at will the horizontal components of magnetism without significant change in the ambient vertical component, which throughout the experimental studies remained the earth's natural one. Furthermore, with the path of the worms being assayed for only a relatively short distance over the number 2 position, and in a plane parallel to that occupied by the magnet, insignificant field-strength differences were present within any given experimental field. The earth's magnetic field is essentially a homogeneous one. The fields that were employed in this study were similarly relatively homogeneous. The field gradient was less than ½ gauss per centimeter.

In those experiments in which the magnet was rotated in a horizontal plane in the earth's field there was a change not only in direction of the imposed horizontal component, but there was also a difference in its strength as the magnet's contribution supplemented or antagonized the geomagnetic one. No attempt was made to compensate for this. For the 10-gauss field, for example, this involved about a 3½% range and for the 5-gauss one, nearly a 7% one. However, these field-strength differences can not alone account for the resolution of the direction of horizontal vector by the organisms since field-strength differences many times larger than these small percentages did not duplicate the influences of small changes in field orientation. Experiments are now in progress which are expected to provide information as to the relative roles of changes in the strength and direction of the horizontal vector.

The problem of resolving organismic responses to weak magnetic fields is compounded by the recent discovery that mud-snails are extraordinarily sensitive to differences in the horizontal vector of electrostatic field (Webb, Brown and Schroeder, 1961). Furthermore, *Dugesia* too has such responsiveness and displays a "compass direction effect" in response to very weak electrostatic fields which is, at least in good measure, independent of the magnetic-compass response (Brown, 1962b). In the present studies, no attempt was made to control the ambient geo-electrostatic field and its changes.

The implications of findings such as the ones reported here, and ones described earlier for the mud-snail, *Nassarius* (Brown, Brett, Bennett and Barnwell, 1960; Brown, Webb and Brett, 1960; Brown, Bennett and Webb, 1960), are great not only in providing an additional parameter to contribute toward the solution of such stubborn problems as those of living clocks and navigational systems of organisms, but also for the problem of regulation within living systems in general. With biological systems possessing astounding sensitivity to such weak magnetic fields, the possibility exists that magnetism may normally play a role in general, organismic integration, either directly or through the biological clock system.

The kinds of magnetic responses described here for *Dugesia* appear not to be specific for this flatworm but simply to represent a general property of living things. The potential of such a sensitivity, with capacity to resolve strength and directional changes, when incorporated into adaptive behavioral systems as an informational input seems tremendous. Search for possible important adaptive roles of these extraordinary biomagnetic sensitivities will probably be very rewarding.

SUMMARY

1. The orientational response of the planarian, *Dugesia,* at a given time of solar day undergoes what appears to be a semi-monthly or monthly fluctuation, probably

a consequence of the possession of a lunar-day rhythm in response to some compass-directional factor.

2. The monthly rhythm in *Dugesia* is modifiable by a weak magnetic field.

3. The monthly rhythm appears to undergo an annual modulation.

4. *Dugesia* exhibits a response to weak magnetic fields in the range of 0.17 to 10 gauss.

5. *Dugesia* differentiates between a horizontal field parallel to the long axis of the body and a field at right angles, and between N and S poles, and, furthermore, is able to resolve intermediate angular orientations of field with remarkable precision.

6. The response of *Dugesia* alters its character in passing from a field close to the earth's strength to one as little as 10 gauss, suggesting the perceptive mechanism to be specifically adapted to such a weak field as the geomagnetic one.

7. There is suggestive evidence that the protozoan *Paramecium* also responds to very weak magnetic fields.

8. Some possible roles for organisms of such astounding responsiveness to very weak magnetic fields are discussed briefly.

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RESPONSE OF THE PLANARIAN, DUGESIA, TO VERY WEAK HORIZONTAL ELECTROSTATIC FIELDS[1]

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A deep-seated, persistent, rhythmic nature, with periods identical with or close to the major natural geophysical ones, appears increasingly to be a universal biological property. Striking published correlations of activity of hermetically sealed organisms with unpredictable weather-associated atmospheric temperature and pressure changes, and with day to day irregularities in the variations in primary cosmic and general background radiations, compel the conclusion that some, normally uncontrolled, subtle pervasive forces must be effective for living systems. The earth's natural electrostatic field may be one contributing factor.

A number of reports have been published over the years advancing evidence that organisms are sensitive to electrostatic fields and their fluctuations. More recently Edwards (1960) has found that activity of flies was reduced by sudden exposures to experimental atmospheric gradients of 10 to 62 volts/cm., and that prolonged activity reduction resulted from gradient alternation with a five-minute period. In 1961, Edwards reported a small delay in moth development in a constant vertical field of 180 volts/cm., but less delay when the field was alternated. The moths tended to deposit eggs outside the experimental field, whether constant or alternating, in contrast to egg distribution of controls. Maw (1961), studying rate of oviposition in hymenopterans, found significantly higher rates in the insects shielded from the natural field fluctuations, whether or not provided instead with a constant 1.2 volts/cm. gradient, than were found in either the natural fluctuating field, or in a field shielded from the natural one and subjected to simulated weather-system passages in the form of a fluctuating field of 0.8 volts/cm.

A study in our laboratory early in 1959 (unpublished) by the late Kenneth R. Penhale on the rate of locomotion in *Dugesia* suggested strongly that the rate was influenced by the difference in charge of expansive copper plates placed horizontally in the air about six inches above and closely below a long horizontal glass tube of water containing the worms. Locomotory rates in fields of 15 volts/cm. (+ beneath the worms) were compared with those in fields between equipotential plates. The fields were obtained with a Kepco Laboratories, voltage-regulated power supply. A comparable study with the marine snail, *Nassarius,* by Webb, Brown and Brett (1959), employing a Packard Instrument Co., high-voltage power supply, confirmed the occurrence of such responsiveness to vertical fields of 15 to 45 volts/cm., and advanced evidence that the response of the snails displayed a daily rhythm.

More recently, it was demonstrated that mud-snails, even while submerged in sea water, were able to resolve a horizontal field difference of 2 volts/cm. in the

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[1] This study was aided by grants from the National Science Foundation, G-15008, and the National Institutes of Health, RG-7405, and by a contract between the Office of Naval Research, Department of Navy, and Northwestern University, 1228–03.

air at right angles to their bodies, and to exhibit a characteristic orientational response (Webb, Brown and Schroeder, 1961). The fields were obtained with B batteries. The snails appeared able also to distinguish the direction of the very weak gradient across their bodies. The character of the electrostatic response was altered simply by changing from South to East the compass direction in the earth's field in which the response was assayed. There seemed to be an influence upon the electrostatic response, by some natural force the effectiveness of which altered with geographical orientation of the organisms.

The following study was made in order to determine whether a comparable sensitivity to very weak electrostatic fields obtains for a common fresh-water planarian, and if so, to learn more concerning its properties.

Methods and Materials

The turning of planarian worms, *Dugesia dorotocephala,* was assayed as they moved forward from an initially enforced orientation in a weak three-light field (Brown, 1962). The three light sources were (1) directly vertical to the initial point in the path, (2) in the horizontal plane directly behind the initially oriented worm, and (3) horizontally 90° to the right of the starting point. In response to this configuration of illumination, the mean path of samples of the worm population, photonegative, always included turning of the worms to the left. The strength of left-turning response was rendered quantitative by recording, to the nearest 5°, the points at which the worms crossed the arc of a circle of one-inch diameter centered at the starting point of the worm. Clockwise turning of the individuals was recorded in positive degrees of arc and counterclockwise turning by negative degrees of arc (Fig. 1) from a mean path directly forward (0°).

The effects of horizontal electrostatic-field gradients were determined by comparing the mean values for 15-path samples in field gradients modified by rendering two aluminum plates in air (Fig. 1), to right and left, equipotential or with potential difference of 45 volts with + to right or + to left. The effects were studied under experimental conditions in which other variables included: (1) magnetic compass direction of the initial, enforced orientation of the worm, which was modified by rotating the whole apparatus to the desired compass direction; (2) time of day the experiment was conducted; and (3) experimental alteration of the natural magnetic field by a horizontal bar magnet centered an appropriate distance beneath the apparatus.

In practice, the worms were placed in a $3\frac{3}{4}$-inch glass Petri dish in 0.5 cm. of water and the dish centered on polar-coordinate paper. This was set upon the floor of a blackened wooden box. The upper portion of one side of the box was open to the observer. In the roof of the box, 16 inches high, was a small light source. The horizontal light sources were onion-skin-covered ends of 10 mm. solid glass tubing, enclosed in black shielding. Through these light was transmitted into the box from a $7\frac{1}{2}$-watt incandescent lamp firmly attached to the outside of the box. Symmetrically to right and left of the Petri dish were large 7 × 9 inch aluminum plates, sandwiched between glass plates darkened with flat black paint. The level of the worm starting-point was close to an axis between the centers (horizontal and vertical) of the two plates. The aluminum plates were about 8 inches apart, thus giving about a 2-inch air space between each plate and the

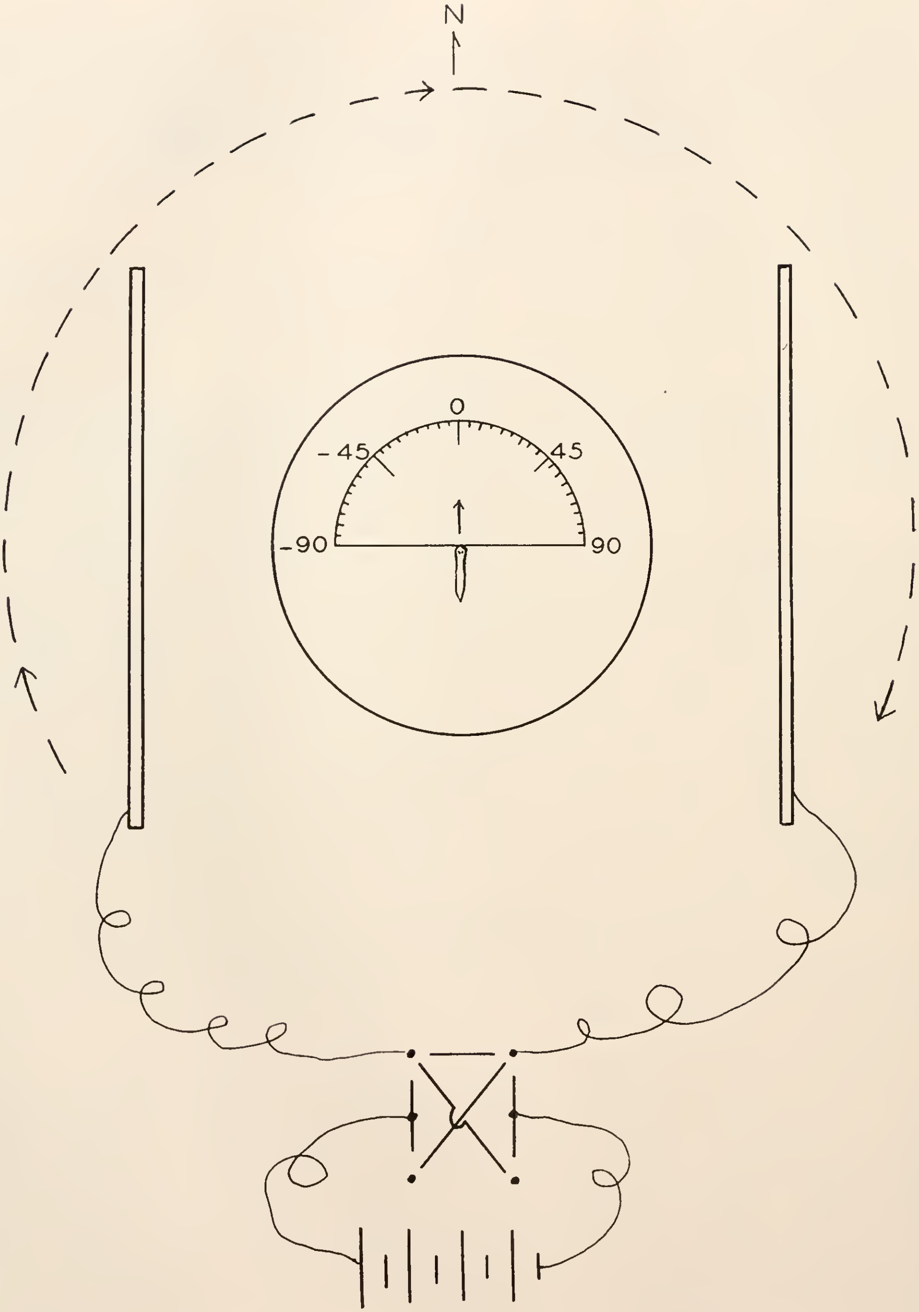

FIGURE 1. The apparatus organization employed in this study, including Petri dish, polar coordinate grid, and aluminum plates. Three-light arrangement is not illustrated. Broken circular line indicates apparatus rotatable relative to earth's geographic field.

Petri dish. The whole visual environment of the orienting worms comprised a rigid system which was constant as the apparatus was rotated in the earth's field. This apparatus was always used in a darkened cubicle to minimize extraneous illumination. As many as four identically constructed pieces of equipment were in operation concurrently, and sequentially, during the course of the study.

The overall average field gradient contributed by the plates was about 2 volts per cm. when these were connected with a 45-volt B battery, with the polarity alterable by a pole-reversing switch. When the battery was disconnected by a toggle switch (SPDT) the plates were simultaneously directly interconnected to assure their equipotential state.

The experiments in any given series were conducted at the same time each day to avoid any complicating factor introduced by a daily rhythm. In addition, each experiment extended over two or more months to randomize any lunar daily influence which might obtain comparable to those well-established to occur for response to very weak magnetic fields.[2]

RESULTS

Response of South-directed worms in the morning: The first series of experiments involved the responses of worms initially always directed magnetic South in the earth's field.The observations were made sometime between 8:30 and 11 A.M. and consisted of two groups of four assays each. Each of the two groups included the determination of the mean paths of 15-worm samples under each of four conditions, two controls (equipotential plates) and two experimentals, + to left (+L) and + to right (+R). The order of the four was selected arbitrarily and differed steadily from one group to the next throughout the two-month experimental period, September 20 through November 17, 1961. The charge across the plates was altered by a person other than the observer. The observer was never informed as to conditions in effect until each day's double series was completed.

The results of this experiment, in which the difference between each, +R and +L, from the mean of the two controls in the group was computed, are plotted in Figure 2. Were the worms incapable of differentiating between the equipotential plates and those possessing a potential difference, the average difference between these would be zero, and the points would be expected to vary randomly about zero. As is evident from inspection of Figure 2, the mean was highly significantly to the right of zero. The mean was $+2.342 \pm 0.342°$ ($t = 6.87$, $N = 152$, $P < 10^{-10}$). These results leave no reasonable doubt that the change from equipotential plates to the 45-volt difference was effecting a mean clockwise turning of *Dugesia*.

The relation of response to compass direction: The foregoing experiment was repeated during the period October 24, 1961, through February 27, 1962, initially by observers different from the one concerned in the first experiment. Five different observers, employing four sets of equipment, eventually contributed to the data. Again, the observations were always made between 8:30 and 11 A.M. But now the effects of the 45-volt difference between the plates, expressed as difference from the equipotential plates, were determined not simply with South-directed apparatus, but with apparatus directed in each of eight compass directions. A single observer on any given day would arbitrarily select one of the compass directions. Otherwise the observations were made just as in the first experimental series. All observers contributed to data from each compass direction. The observers were now informed, however, as to the experimental conditions obtaining, but four of the five observers were uninformed of previous work, or even of the nature of the problem. The results of the four-month study are summarized in Table I, and the frequency distribution for each compass direction illustrated in Figure 3.

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[2] The author wishes to acknowledge here his appreciation to a number of persons, particularly Young H. Park, Sam D. Park, Polly Merrill, Stephanie Struggles, and Gertrude L. Siegel, who devoted many hours to acquiring the data for this study.

There was reasonably good confirmation of the earlier South-directed "uninformed" experimental series. However, a clear compass-direction effect was now evident. When the apparatus was southerly directed, the increase in potential gradient turned the worms clockwise, when northerly directed, counterclockwise, with graded differences between these directions. More particularly (see Figure 4), the results suggested that the axis of the compass-direction effect was a S-SE to N-NW one instead of a magnetic N-S one.

Relationship of response to time of day: While this last experimental study was under way data were being gathered from occasional comparable series run between 2 and 6 P.M., commencing on September 15, 1961. These provided a strong

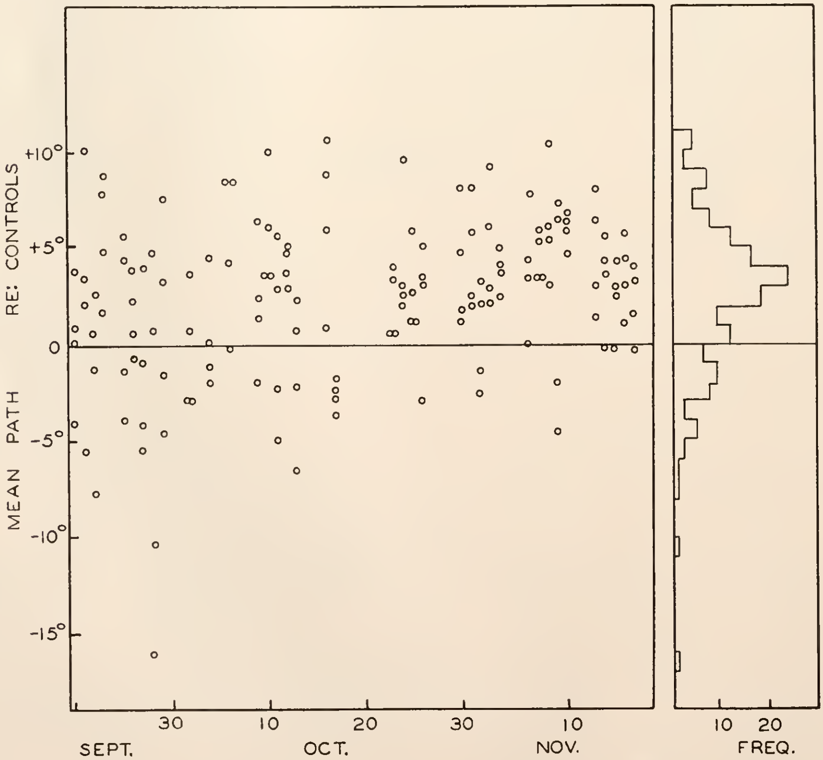

FIGURE 2. The differences between path of worm samples (15 paths) in a 2-volt/cm. right-angle gradient and path in the same experimental series in an equipotential field.

TABLE I

Morning paths

Direction	Mean	S.E.	t	N	Probability
N	−1.325	±0.605	2.13	64	<.05
NE	−1.125	±0.354	3.09	76	<.005
E	+1.330	±0.433	2.92	56	<.005
SE	+1.950	±0.498	3.92	84	<.001
S	+1.805	±0.549	3.29	86	<.005
SW	+2.275	±0.476	4.74	80	<.001
W	−0.920	±0.502	1.41	78	<.20
NW	−1.350	±0.424	3.18	80	<.005

suggestion that afternoon values were not showing the same form of compass-direction relationship as the morning ones. Instead, the results suggested that there was an inversion of the compass-direction effect. This question was eventually pursued more systematically and studied until May 1, 1962. It is evident from Table II and Figure 4 that for two directions for which moderately extensive data were obtained, NW and S, the electrostatic field effect shows a clear inversion of the afternoon values relative to the morning ones. That the afternoon responses for each direction were different from the morning ones was far more clearly apparent

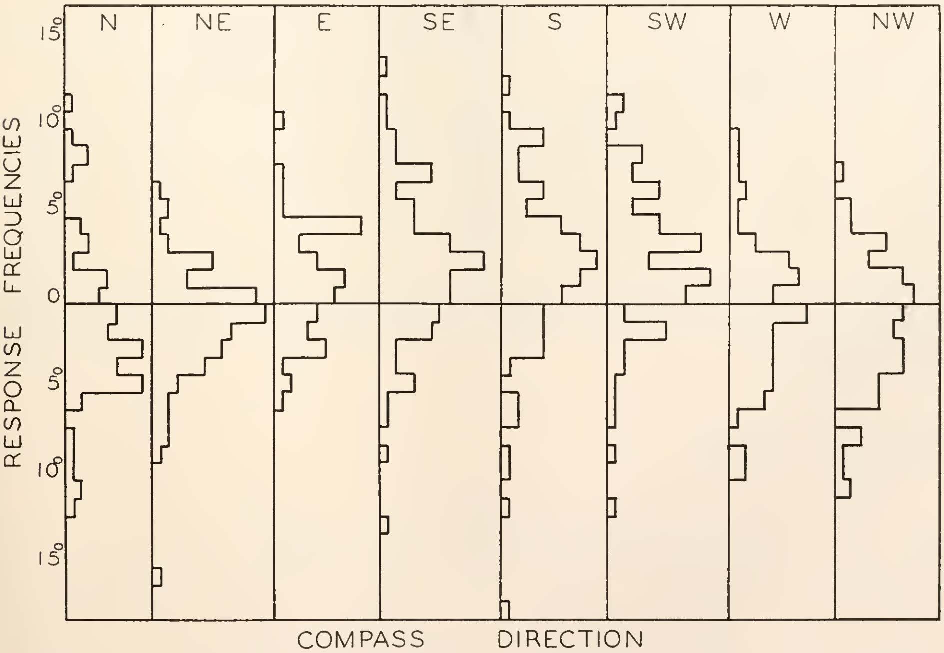

FIGURE 3. Frequency distributions of the differences between paths of worm samples in a 2-volt/cm. right-angle gradient and path in the same experimental series in an equipotential field for each of eight compass-directional orientations.

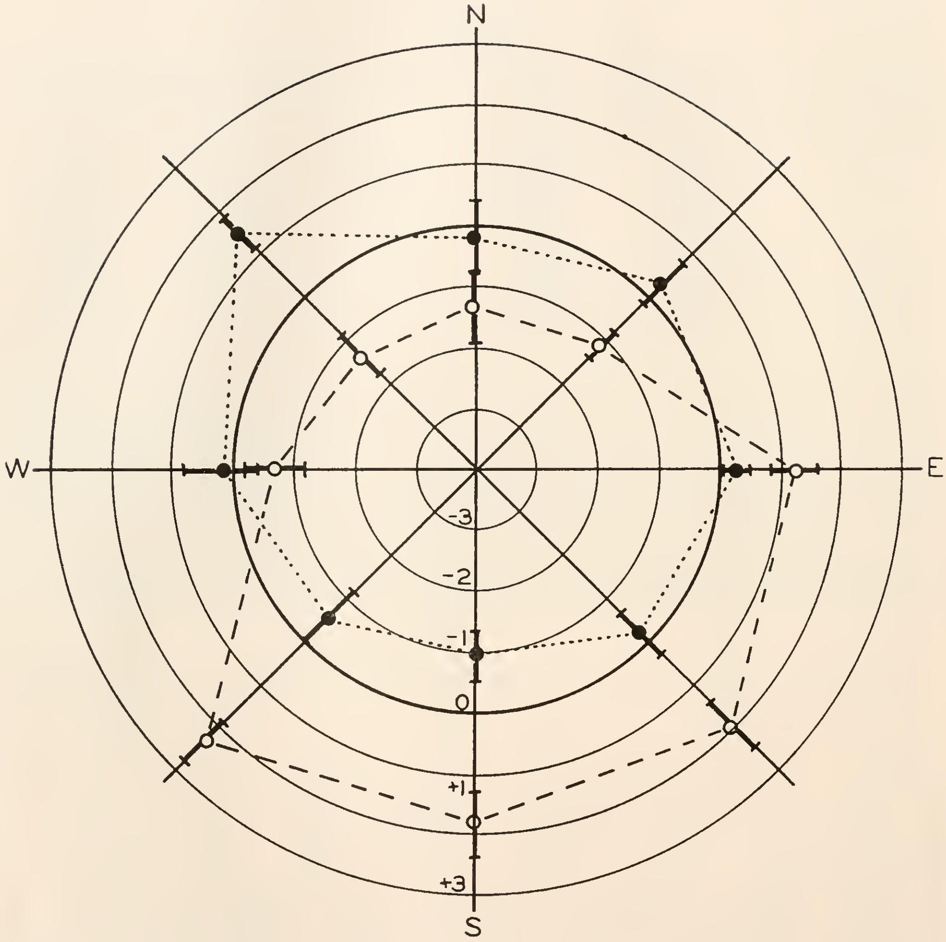

FIGURE 4. Comparison of the compass-direction effect upon response to electrostatic gradient for morning (dashed line) and afternoon (dotted line) hours. Degree of left turning is indicated by concentric circles inside heavily inked one, and right turning by concentric circles outside of it.

upon finding 6 of the 8 directional differences (NE, E, SE, S, SW, and NW) statistically significant.

Influence of experimental magnetic-field changes on response: Since there was conspicuously a compass-directional relationship of the character of the response to the 2 volt/cm. change in electrostatic field it became of interest to learn whether this was directly dependent upon responsiveness of the worms to the magnetic field. Consequently, an additional experiment was conducted between February 17 and April 11, 1962. This comprised, until March 13, observing in the morning the effects upon mean paths of the electrostatic-field difference for North- and South-directed planarians and for North-directed ones under experimental conditions of an artificial magnetic field differing from the earth's only in that the natural 0.17-

Table II

Afternoon responses

Direction	Mean	S.E.	t	N	Prob.	Difference from A.M.	S.E. diff.	t	N	Prob.
N	− .12	±0.48	0.25	42	<.9	+1.20	±0.772	1.55	106	<.20
NE	+ .37	±0.53	0.70	51	<.5	+1.52	±0.634	2.40	127	<.02
E	+ .33	±0.25	1.32	178	<.2	−1.00	±0.499	2.00	234	<.05
SE	− .20	±0.46	0.17	42	<.9	−2.15	±0.678	3.17	126	<.005
S	− .92	±0.46	2.00	62	=.01	−2.73	±0.716	3.81	148	<.001
SW	− .57	±0.54	1.05	40	=.3	−2.85	±0.720	3.96	120	<.001
W	+ .12	±0.56	0.21	41	<.30	+1.04	±0.752	1.38	108	<.20
NW	+1.44	±0.38	3.79	60	<.001	+2.79	±0.569	4.90	140	<.001

gauss North-directed horizontal component of the field was experimentally reversed to become a 0.2-gauss, South-directed one. Upon the basis of response to magnetism, the worms should now receive stimulation closely similar to that normally experienced by South-directed worms. On March 13, one additional condition was added to the series, namely South-directed worms in the earth's field were given experimentally (as a 0.2-gauss field) essentially the magnetic equivalent of a North-directed route.

In practice, each series comprised, in random order, pairs of observations under each of the three or four conditions, with the order, equipotential to non-equipotential plates, in each pair determined by the flip of a coin. The three observers of the worms were uninformed of the order for each pair.

The results of this experiment are shown in Table III. The experiments showed the same qualitative difference between North and South in the earth's field that had been observed in the earlier series similarly conducted in the morning. Evident from the table is the fact that compass-North-directed worms given a South-directed experimental magnetic field, $N_{(S)}$, did not come to behave like South-directed ones, S. Indeed, the experimental reversal of the magnetic field even augmented the characteristic counterclockwise, North-directed response, N. The difference between N and S responses was 1.496 ± 0.546° ($t = 2.74$). The difference between $N_{(S)}$ and S responses was 2.68 ± 0.522° ($t = 5.13$). In fact, the difference between N and $N_{(S)}$, 1.16 ± 0.501°, was itself statistically significant ($t = 2.31$).

The difference between S and $S_{(N)}$ is, unlike the difference between N and $N_{(S)}$, in the direction to be expected were the compass-direction effect to result

Table III

Influence of experimental magnetic-field reversal

Direction	Mean	Standard error	t	N	Probability
N	−0.493	±0.372	1.325	93	<.2
$N_{(S)}$	−1.65	±0.336	4.91	93	<.001
S	+1.03	±0.400	2.57	93	<.02
$S_{(N)}$	−0.22	±0.524	0.42	50	<.7

from an influence of magnetism. Although the sample of $S_{(N)}$ is only about half the size of the other three experimental conditions, due to the late addition to the series, the difference of 1.25 ± 0.658° suggests that a comparable statistically significant difference would have been demonstrated had the sample been larger.

It is clear that experimental reversal of the magnetic field did not reverse the relative differences in path as did a change in compass direction. The introduction of the weak magnetic fields for each of the two directions simply displaced the response to the weak electrostatic field, either to a less positive orientational one (S) or a more negative one (N). In other words, the effect of the reversed magnetic fields appeared to be simply to displace about 1° to the left the electrostatic response-pattern related to compass directed without altering its form. Therefore, this particular compass-direction effect of electrostatic response is in large measure independent of the previously described geomagnetic compass-response of snails and planarians (Brown, Bennett, and Webb, 1960; Brown, 1962).

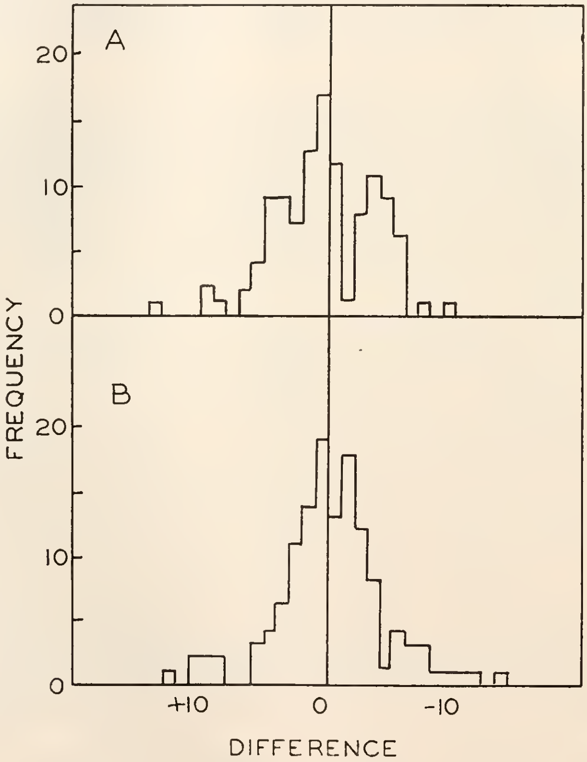

FIGURE 5. Comparison of the frequency distributions of the +R effect minus +L effect for (A) the period November 20, 1961 through March 31, 1962, and (B) the periods September 15 through November 17, 1961 and April 1 through May 1, 1962.

Resolution of field direction across body: The data were searched for evidence concerning whether or not *Dugesia* was able to distinguish between a potential difference with positive charge to right and one with positive charge to left. Assuming no capacity of the worms to distinguish between these two conditions, the difference between the two should average zero, and there should be a random distribution of values about zero.

When, however, the morning differences between the effects of the two field orientations, +R minus +L, were examined for all eight compass directions for the whole period of study, October 24 through February 27, a difference of $+0.821 \pm 0.277°$ was obtained ($t = 2.96$; $N = 297$; $P < .005$). Furthermore, within this four-month period the data departed significantly in both directions from a random distribution in time. For example, from December 4 to 23, inclusive, the mean was $-1.31 \pm 0.51°$; $N = 44$, $P < .02$; whereas from December 26 through February 10, it was much more strongly significant and positive $+1.535 \pm 0.408°$; $N = 170$, $P < .001$. The data suggested that for the former period the worms were distinguishing between the two directions of the field and turning away from +R, and during the latter, were distinguishing and turning toward the +R.

A second kind of suggestion that the worms were able to distinguish the two field-directions came from a comparison of the values of the differences between effects of the two fields, +R minus +L, for the morning and afternoon experimental series. The mean difference computed from all data for the afternoon indicated they were turning away from +R, though not statistically significantly so. When the frequency distributions of all the afternoon values for the colder months (November 17 through March 31) were plotted, a bimodality was suggested (Fig. 5A). It thus appeared again that two kinds of response were evident to +R. There was either (1) a tendency to turn weakly toward it, or (2) a tendency to turn more strongly away from it.

This bimodality was significantly less apparent for all the afternoon values obtained for warmer months (September 15 through November 15, 1961; April 1 through May 1, 1962); the frequency distribution for these two periods is shown in Figure 5B. A Chi-square test for significance of a difference between the two populations of values depicted in Figure 5 gave $\chi^2 = 32.05$, when scattered peripheral values were combined to render $N = 12$, or $P < .003$. Such a difference would not be expected unless +R and +L could elicit different responses by the worms. A suggestion of the occurrence of two signs of responses was present also in the frequency distribution of the morning data.

A third kind of evidence pointing to ability of the worms to distinguish between the two field orientations came from a study of differences in variances of the values

TABLE IV

Variances of +R effect minus +L effect

Direction	Var. through May 1	N through May 1
N	21.85	49
NE	23.20	59
E	13.54	111
SE	17.71	63
S	22.86	150
SW	27.20	58
W	21.45	52
NW	12.95	72

of +R effect minus +L effect, with compass direction for all data. These are presented in Table IV. Variance differs in a statistically significant manner with compass direction. It reaches its highest values in the SW- and NE-directed series with minima for E and NW ones. Significance is readily demonstrable by the F test for differences between variances, between minima and maxima in this compass-direction effect (*e.g.*, E to NE, $P < 0.05$; E to SW, $P < .01$; NW to SW, $P < .01$; NW to NE, $P < .05$). It is self-evident that such differences with compass direction could not be expected were +R to be physiologically indistinguishable from +L.

The evidence, taken as a whole, suggests therefore that the relative responses of the worms to +R and +L vary with time, with geographic orientation of the worms, and with hour of the day.

Discussion

In the study which is reported here, the exact values of the fields to which the animals were subjected were never known. The natural field was unquestionably reduced substantially and maximally in the horizontal axis connecting the two equipotential plates, and minimally in all axes at right angles to this, including both horizontal and vertical ones. The important thing for this study was that whatever horizontal potential gradient remained at right angles to the initial path of the worms, the experimental gradient in one direction added 2 volts per cm. to that field, and in the other direction subtracted this amount. By such means it was possible, therefore, to determine whether the animal could resolve such small changes.

The orientation of the worms in the experiment was observed while they were submerged in tap-water whose source was Lake Michigan. Such water is, relative to the surrounding air, a good conductor. Therefore, the overall electrostatic gradient to which the worms were directly subjected was far smaller than the 2 volts/cm. gradient in the air. The value can be estimated to be 6 to 8 orders of magnitude below that in the air as a consequence of the "Faraday-cage effect" of the worm's ambient aqueous medium. To exhibit such responses as the worms did in these experiments would require a sensitivity to essentially static electric gradients of the order of fractions of a microvolt per centimeter.

The significance of this demonstrated sensitivity for animals is apparent. In speculations on the mechanism involved in the reported responses of insects to atmospheric gradients, surface charge has been importantly considered (Edwards, 1960). In the light of the "Faraday-cage action" of every organism's body as it behaves as a volume conductor, it has been difficult to believe that the minute residual gradients within the organism, correlated with the larger atmospheric gradients, could result in any response of individual cells or organs located protectively inside the external boundaries of the organism. The studies with the worms, and earlier studies with the marine snails submerged in sea water (even a slightly better conductor), have proven there exists cellular sensitivity adequate to require a reconsideration of the mechanism of response in such terrestrial organisms as the insects, hamsters (Schua, 1954) and even man (Frey, 1952).

Sensitivities of the order of those established by this study provide one means for an influence of weather-system changes on organisms. Such meteorological changes are not uncommonly accompanied by electrostatic fluctuations more than one hundred times as great as the experimental ones employed in this study. An innate ability of living things to interpret specific parameters of electrical change in their environment may prove to be a partial explanation of apparent forewarnings some organisms have appeared to receive relative to meteorological disturbances.

Ability to resolve small differences in strength of horizontal vectors of atmospheric electrostatics, and their direction as well, can contribute as a navigational aid. This would comprise an electrostatic "compass." Such a compass may be used along with other aids, such as response to magnetic field and visual responses, including use of celestial references.

The earth's atmosphere displays periodic variations in diverse electrical parameters. These relate importantly to movements to the earth with respect to sun and moon. Ability to resolve strength, direction, and frequency and amplitude of oscillations in electrostatic field, can theoretically provide an organism with a means of deriving valuable information as to the period lengths of the natural geophysical rhythms. Both local-time and universal-time components are present in these fluctuations. Responsiveness to electrostatic fields may possibly be one of the normally contributing factors to the timing system of the extraordinary clocks of animals and plants.

Such sensitivity of a protoplasmic system to an electric field as appears to be present renders it probable that protoplasm is far more sensitive to electromagnetic fields of radio-frequency than has generally been conceded, or even reported, up to the present. This possibility is further supported by the correspondingly great sensitivity to extremely weak magnetostatic fields reported elsewhere. It is conceivable that failure to disclose such perceptivity may commonly be a consequence of an inability, to date, to discover an invariable kind of response by the organism to such a stimulus.

The complexity of the response mechanism of the planarians to electrostatic fields as revealed by these studies, and the relationships of the response to both temporal and spatial orientation, certainly suggest the hypothesis that responsiveness to this factor plays still undisclosed and important roles in the lives of terrestrial creatures.

SUMMARY

1. The planarian *Dugesia* is able, even while in water, to perceive a change of 2 volts/cm. in electrostatic gradient in the surrounding air.

2. There is reason to presume that in order to show this response the organism is responding to differences in ambient static gradient of the order of fractions of a microvolt per cm.

3. The strength and character of worm response to a right-angle potential change are related to the direction the worm is oriented in the earth's geographic field, and to time of day.

4. A field-change in South-bound worms in the morning effects clockwise turning. A similar field-change for North-bound worms effects counterclockwise turning. In the afternoon the relationship of electrostatic response to geographic direction is essentially the mirror-image of that of the morning.

5. *Dugesia* is able to distinguish the direction of a gradient across its body.

6. A few of the possible significances of these findings are discussed briefly.

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FREE GLYCEROL IN DORMANT CYSTS OF THE BRINE SHRIMP ARTEMIA SALINA, AND ITS DISAPPEARANCE DURING DEVELOPMENT[1]

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During part of a previous study on the stored carbohydrates of various dormant organisms (Clegg and Filosa, 1961), large amounts of a carbohydrate-like substance were observed in extracts of *Artemia salina* cysts. On the basis of mobility and reactivity on paper chromatograms, this substance appeared to be glycerol. Since large amounts of free glycerol have been shown to accumulate during diapause, and in other hypometabolic stages of certain insects (*cf.* Salt, 1961), a more thorough analysis was undertaken. In addition, although several studies have been made of the chemical components of these cysts, no mention has been made of glycerol (the extensive literature on *Artemia* has recently been cited by Dutrieu, 1960). The present report deals chiefly with the identification of free glycerol in *Artemia* cysts and the changes in its concentration during development. A preliminary report on the presence of glycerol in *Artemia* cysts has been published (Clegg and Evans, 1962).

Materials and Methods

Dried cysts of *Artemia*, which are embryos in the early stages of development covered by a chitinous shell (Dutrieu, 1960), were obtained as a gift from the Brine Shrimp Sales Co., Inc., Hayward, California. Unless designated otherwise, the cysts used were collected in the fall of 1960 from the evaporating ponds near Hayward, and analyses of these cysts were begun in the summer of 1961. They were washed briefly with distilled water to remove any empty shells, and were then dried at room temperature for at least twenty days before use. Over 70% of these cysts produced active nauplii when incubated in sea water at 24–26° C.

For the isolation of glycerol, about 5 g. of cysts were homogenized in a Ten Broeck homogenizer with 30 ml. of 80% ethanol. The homogenate was filtered and the filtrate decolorized with Norit (1% w/v). After removal of the Norit, the clear filtrate was concentrated under reduced pressure and then extracted with benzene. The organic phase was discarded and the remaining solution was purified by paper chromatography (Evans and Dethier, 1957). The combined eluates from the chromatographic separation were concentrated by evaporation at 50° C., and then dried over $CaCl_2$ to a viscous syrup (about 300 mg.) which then was used for the identification studies.

For quantification of glycerol, 40–80 mg. of cysts were homogenized in 1.0 ml. of 80% ethanol; the homogenate was transferred to a graduated centrifuge tube

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with four 1-ml. washings, and the volume was made up to 5.0 ml. with distilled water. After centrifuging for 15 minutes at 3000 rpm., aliquots were taken for determination of glycerol in the supernatant by the colorimetric method of Lambert and Neish (1950) as modified by Burton (1957). In preliminary experiments, the glycerol was first isolated and identified by paper chromatography and then eluted for quantification. It was found later that results obtained by this method differed from direct determinations on the supernatant by not more than 3%. As a result, direct colorimetric determinations were carried out on the supernatant.

Trehalose was determined by the anthrone method of Dimler *et al.* (1952) after its isolation from the supernatant by paper chromatography (Clegg and Evans, 1961). The pellet from the ethanol extraction was analyzed for polysaccharide by re-homogenizing in 5.0 ml. of 5% trichloroacetic acid, centrifuging, and using an aliquot of the supernatant for the anthrone method of Dimler *et al.* (1952). Results obtained by this procedure were similar to those obtained by conventional alkali extraction and alcohol precipitation methods. This material, when hydrolyzed by acid, yielded only glucose, as judged by paper chromatography, and will be referred to as glycogen in the present study.

To obtain the nauplii, a known amount of cysts (40–80 mg.) was incubated in a Petri dish containing filtered sea water at 24–26° C. In all cases, the nauplii were collected within three hours of emergence from the cysts and were separated from the mixture of developing cysts and shells by virtue of the fact that the nauplii were positively phototactic while the cysts and shells floated on the surface. The nauplii were pipetted from the medium, filtered, and washed with distilled water. They were then dried to constant weight and analyzed by the same methods given for the cysts. The empty shells were collected and analyzed after 96 hours of incubation. Average weights of the cysts, shells, and nauplii were obtained by placing 50 to 100 individuals on a pre-weighed coverslip, drying to constant weight, and re-weighing on a Mettler Micro Balance (sensitive to about 1 μg).

Results and Discussion

Identification of glycerol

The substance in question migrated on paper chromatograms with authentic glycerol in the following solvent systems (v/v): (1) water-saturated ethyl acetate; (2) n-butanol, ethanol, acetone, water (5:4:3:2); (3) chloroform, ethanol (8:2); (4) ethyl acetate, ethanol, water (12:2:1); and (5) n-propanol, ethyl acetate, water (7:1:2). When mixtures of the substance and authentic glycerol were chromatographed, no separation was observed in these solvent systems. Positive identification of the substance as glycerol was established by preparation of the tribenzoate derivative (Segur, 1953). The product, recrystallized from 90% ethanol, had a melting point of 71–72.5° C. The tribenzoate prepared from authentic glycerol had a m.p. of 71.5–72.5° C., and the mixed m.p. was 72–73° C.

Levels of glycerol and glycogen in the cysts and nauplii

Dutrieu (1960) has shown that net glycogen synthesis occurs in *Artemia* during the transition from the dormant cyst to the active nauplius. Net glycogen synthesis also occurs after diapause is broken in the eggs of the silkworm, *Bombyx mori* (Chino, 1957), and glycerol and sorbitol were shown to be its precursors (Chino,

TABLE I

Glycerol and glycogen content of the cysts, and nauplii of newly emerged Artemia

Stage	Per cent of the dry weight			
	Glycerol		Glycogen	
	Mean ±S.E.	No.	Mean ±S.E.	No.
Cyst	4.91 ± 0.42	(9)	1.13 ± 0.09	(8)
Shell	0.19	(3)	0.04	(3)
Embryo	6.30* ± 0.48	(9)	1.86* ± 0.14	(8)
Newly emerged nauplius	4.85 ± 0.21	(13)	15.1 ± 0.2	(6)

* 1 mg. cysts = 0.78 mg. embryo (Table II); 4.91 ÷ 0.78 = 6.30% glycerol of the embryo dry weight.

1958). Therefore, studies were undertaken to determine whether or not glycerol was converted to glycogen following the termination of dormancy in *Artemia.* Sorbitol, incidentally, was not found in these cysts (limit of detection = 0.2% of the dry weight).

Table I summarizes the results obtained by incubating cysts in 2% NaCl. Glycerol was present in the dried cysts before incubation to the extent of about 5% of the dry weight and, on the basis of cyst dry weight, no decrease was measured during the transition from cyst to nauplius. Similar values were obtained by homogenizing the cysts in distilled water at 0–4° C. This indicated rather strongly that the amount of glycerol found was present as free glycerol in the cyst. The small values given for the shells are maximal since a few undeveloped cysts might also be present in the shell fraction. In addition, it should be pointed out that the assay system used is not wholly specific for glycerol, so these low values may not be glycerol at all. In any event, it was clear that most, if not all, of the glycerol was confined to the embryo. In order to compare the glycerol levels in the embryo with those in the nauplius it was first necessary to estimate the weight of the embryo. This was so because the shell, constituting a large percentage of the cyst weight, was shed when the nauplius emerged, and would not be used as a basis for estimating glycerol levels in the nauplius. This information, given in Table II, indicated that the embryo constituted about 78% of the cyst dry weight. This figure was then used to calculate the concentration of glycerol in the embryo, shown in Table I as over 6% of the dry

TABLE II

Dry weights of the cyst, shell, and nauplius

Stage	Mean weight		Per cent of the cyst weight
	μg. ±S.E.	No.	
Cyst	2.55 ± 0.03	(7)	100
Shell	0.57 ± 0.01	(7)	22
Embryo	1.98 ± 0.03	(7)	78
Newly emerged nauplius	1.93 ± 0.16	(8)	—

weight. Even on this basis the glycerol content decreased by only about 1.5% of the dry weight during the formation of the nauplius. At the same time, glycogen levels increased by about 14% of the weight, on the basis of cyst and embryo dry weight, as shown in Table I. Clearly, the small decrease observed in the glycerol content could not account for the amount of glycogen synthesized. Therefore, the source of most of this glycogen, unlike *Bombyx* eggs, was not glycerol. It would appear from the study of Dutrieu (1960) that trehalose, a non-reducing disaccharide of glucose,

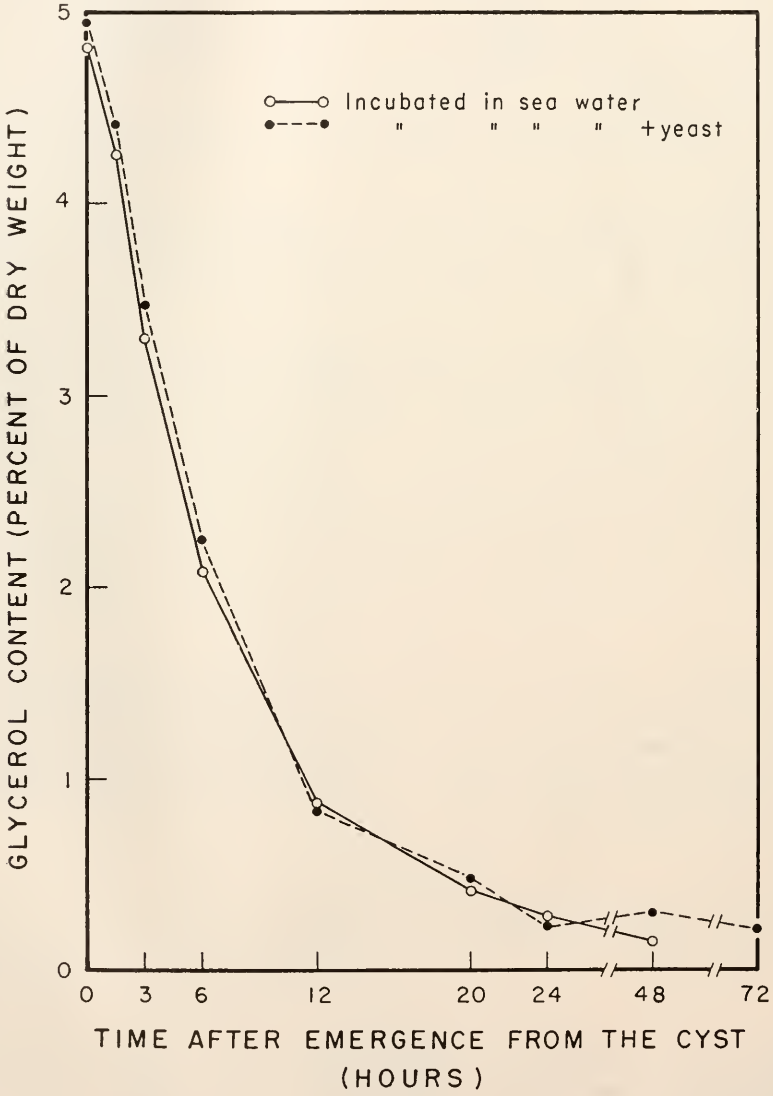

FIGURE 1. Glycerol content of fed (●) and unfed (o) nauplii as a function of time after emergence from the cyst.

might be the chief substrate for the glycogen synthesis observed during the development of *Artemia*. This aspect will be considered in a future publication. The values given in Table I for glycogen concentrations in the nauplius are more than twice those reported by Dutrieu (1960). There are several obvious possible explanations for this difference.

The results given above indicated that glycerol was not being used either as an important source of energy during development or as a major substrate for glycogen synthesis. Accordingly, the fate of glycerol in the nauplius was examined.

Glycerol levels as a function of nauplius age

A large number of newly emerged nauplii was collected as described and divided into two groups. One group was incubated in filtered sea water and the other in sea water containing 1 mg. of dried yeast per ml. as a source of food. After various periods of incubation the nauplii were analyzed for glycerol content. The averaged results of three separate experiments are given in Figure 1. The amount of glycerol in the nauplii decreased sharply during the first 24 hours of incubation and then remained at a very low, constant level. These latter values are probably due to the presence of small amounts of non-glycerol substances that produce color with the reagents, since glycerol could not be detected in 72-hour-old nauplii when these extracts were analyzed by paper chromatography (limit of detection = 0.2% of the dry weight). Since the rapid decrease in glycerol content was observed in fed and unfed nauplii it seems that glycerol disappearance is not influenced by nutrition. Comparisons of the glycogen content of these two groups of nauplii were not made since, in the case of those incubated with yeast, the amount of glycogen present in the gut lumen, due to the presence of ingested yeast, was uncontrollable. Consequently, it is not known whether the decrease in glycerol is accompanied by an increase in glycogen. Because the nauplii are so small, attempts have not yet been made to follow the metabolic fate of injected radioactive glycerol. The present results do show, however, that glycerol essentially disappears during the first day following emergence from the cyst.

Glycerol, trehalose, and glycogen contents of aged cysts

Next, the effect of source, age, and storage condition on the carbohydrate composition of the cyst was examined. These aged cysts, and a brief resumé of their history, were generously supplied by Mr. Maurice Rakowicz of Brine Shrimp Sales Co., Inc., Hayward, California. At least 200 mg. of cysts from each group were analyzed for trehalose, glycerol, and glycogen content by the methods described above. Dutrieu (1960) has shown that trehalose and small amounts of glucose are the main alcohol-soluble sugars present in *Artemia* cysts and this has been confirmed in the present study. The per cent hatch was obtained by incubating at least 500 cysts from each of the groups for 72 hours in sea water at 24–26° C., and then counting the number of viable nauplii produced. The results, summarized in Table III, showed that the trehalose content of these several groups was quite constant, whereas the glycogen and glycerol contents showed considerable variation. The most striking difference between these groups was the per cent hatch, none of the cysts producing viable nauplii in the 1938 group. Noteworthy was the increased viability of those cysts stored *in vacuo* compared with those stored in air since 1951.

TABLE III

Glycerol, glycogen, and trehalose contents of aged cysts

Origin of cysts and date collected	Storage	Per cent of the dry weight			Average % hatch
		Glycerol	Trehalose	Glycogen	
San Francisco, 1961	air	4.91	14.27	1.13	73
San Francisco, 1951	air	2.48	16.49	1.18	5
San Francisco, 1951	vacuum	2.49	17.29	1.65	62
San Francisco, 1938	air	2.45	14.68	1.05	0
Great Salt Lake (Utah), 1951	air	4.73	15.09	2.67	4

The fact that cyst viability greatly decreased with aging in air, while the trehalose and glycogen content did not appear to diminish appreciably, suggests that a source of energy is not the limiting factor determining viability during aging for long periods. These findings also emphasize the "metabolic dormancy" of these cysts, at least with respect to carbohydrate metabolism. For the present, however, the main conclusion derived from these results was that trehalose, glycogen, and glycerol are the normal and principal carbohydrates of dormant *Artemia* embryos. A detailed study is presently being made to determine the origin of glycerol in the embryo, the metabolic fate of glycerol in the nauplius, and the role of glycerol and trehalose in the dormancy of *Artemia* cysts.

I express my thanks to Dr. David R. Evans for a critical reading of the manuscript.

SUMMARY

1. Free glycerol was identified as a major carbohydrate component of the dormant cysts of *Artemia salina.*

2. The amount of glycerol present in cysts aged for a year in the dry state was found to be about 5% of the cyst weight, and was shown to be restricted to the embryonic part of the cyst.

3. Glycerol content decreased slightly during the formation of the nauplius and then rapidly decreased to a very low level after the nauplius emerged from the cyst. The decrease in glycerol content could not account for the synthesis of glycogen during formation of the nauplius.

4. The glycerol, trehalose, and glycogen contents, and the viability of cysts aged up to 28 years were determined.

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THE SURVIVAL OF ARTEMIA POPULATIONS IN RADIOACTIVE SEA WATER

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With salt-water organisms there have been few attempts to check conclusions based on data from the more traditional species used in radiation genetics. Possibly this traces to difficulties in maintaining stocks of known ancestry, although we have met no serious maintenance problems with strains of *Artemia*. The brine shrimp thrives without running water and thus we avoid escape of zygotes or the loss of floating gametes. Furthermore, their ability to cope with environmental stresses, including ionic and osmotic changes (Lochhead, 1941), suggested that *Artemia* would be ideal for experiments in which radioisotopes would be added to sea water.

The persistence of mass cultures and the fitness components obtained from pair mating tests are reported below for *Artemia* whose ancestors survived sea water to which either P^{32} or Zn^{65} had been added. These isotopes were used because radiation ecologists have shown particular concern about their presence in the vicinity of nuclear reactors and atomic test sites (Gong, *et al.*, 1957; Davis, 1958; Davis *et al.*, 1958). For comparison and contrast, data from experimental populations whose ancestors had received acute exposures to x-rays are also included.

Considering fitness to be measurable as the number of mature offspring left by tested parents, we obtained a basis for comparison between descendants of control and irradiated *Artemia*. There is no evidence of increased fitness over controls for any experimental population in sea water, diluted sea water or brine.

MATERIALS AND METHODS

Stock origin and maintenance

Our *Artemia salina* stocks originated from commercial dry cysts of the diploid amphigonic California strain. Although mass culture techniques were explored earlier (Grosch and Erdman, 1955), the oldest cultures extant date from 1957. One of these, number 3 used in the present study, has been maintained from its beginning in the same 5-gallon rectangular battery jar. Additional available control cultures were begun in 1959 in the same cylindrical gallon jars now containing them. Of the several available, number 8 has been used in the present study. Control maintenance has not been a problem. In fact, five control cultures have been discarded due to limitations in space. All control cultures were started from several

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hundred dry cysts, and as many as 300 well developed *Artemia* have been counted in a gallon control at the height of the summer. Earlier, in June, the first group to mature tends to be somewhat smaller, numbering 50 to 100.

Using ten pairs of adults per three liters of sea water seemed the most feasible approach to setting up radioisotope experiments. A series of doses can be instituted simultaneously without endangering persistence of the control culture from which the pairs of adults are removed. Culture #3 provided the parents for all experimental cultures to date. Table I summarizes these cultures and the nature of their treatments.

Table I

Inception and subsequent history of three-liter experimental cultures of Artemia. T = tested by pair matings. DNS = did not survive. ? = survival questionable

1958	1959	1960	1961	1962
μc. P^{32} added				
30 A	T	T	T	testing
B	T	T	too few to test	DNS
	30 μc. P^{32} added	DNS		
		30 μc. P^{32} added	DNS	
			30 μc. P^{32} added	
40				
60				
90	T	T	T	DNS
120	DNS			
	μc. P^{32} added			
	30			
	90	too few to test	T	testing
	120	became extinct		
	150	DNS		
	200	DNS		
	450	DNS		
	r, x-ray			
	1000	T	T	testing
	2500	DNS		
	3000	DNS		
		r, x-ray		
		1000		
		2500	DNS	
		3000	DNS	
		μc. Zn^{65} added		too few to test
		30	T	
		60	DNS	
		90	DNS	
		120	DNS	
			μc. Zn^{65} added	
			30	?
			60	DNS
			90	DNS
			120	DNS

In 1958, P^{32} in phosphate form was added to a series of three-liter (3-L.) cultures at the following levels: 30, 40, 60, 90 and 120 μc. The 30 μc./3-L. culture gave rise to two subcultures known as "A" and "B" which differ by one generation. In August, when F_3 larvae became evident, the F_2 parents were removed to another 3-L. jar where they produced cysts which overwintered. This culture was designated "A." The culture derived from the cysts produced by the F_3 remaining in the original jar has been known as "B."

In 1959 duplicate experiments were set up at 30, 90, and 120 μc./3-L. In addition higher doses were given to check on the suspected limits of tolerance: 150, 200, and 450 μc. In 1959 and each successive year descendants from the 1958 30-μc. dose were subcultured and given a repeated dose of 30 μc. of P^{32}

Zn^{65} in chloride form was added to four 3-L. cultures in 1960 at the following levels: 30, 60, 90, and 120 μc. This was repeated in 1961.

The x-ray exposures were made in 1959 and 1960. Each year, ten pairs of adults were given three doses each from the Woods Hole generator. It operated at 30 ma. and a 200 Kv. peak with an inherent filter equivalent to 0.2 mm. of Cu. Delivered in a few minutes, the acute doses were 1000 r, 2500 r, and 3000 r, respectively below, near and above the dose found sterilizing for adult females (Grosch and Erdman, 1955; Grosch and Sullivan, 1955). All cultures have remained indoors, shelved near, but not in, windows which receive sunlight for half of the day. The cultures were untouched from September until June. During this winter period, the water gradually evaporated until only an inch of saturated brine remained, along with crystalline salt deposits and colorless algal debris. Persistent adults were seen only occasionally. The cultures were reconstituted from the cysts deposited on the sides of the container upon filling with distilled water to the original high water line. The salts dissolved with stirring.

In general the procedure followed a natural sequence of events described by Boone and Baas-Becking (1931) for California salterns, where "winter eggs" are left along the high water marks, to "swell and burst" in the spring when freshets dissolve the salt crust and the environment reaches a favorable salinity. In laboratory cultures it has seemed necessary to remove any large masses of putrefying algae soon after emergence of the *Artemia* larvae. Earlier removal may not be advantageous because some cysts can be trapped in the mass.

Pair mating tests

In order to study reproductive capacity and adult life span, pairs of young adults were moved to quart jars from the large mass cultures upon reaching sexual maturity. Transfer was by dipping or pouring because adults are easily injured by pipetting. The pair matings were inspected daily until the death of both animals. Upon their appearance, broods of live young were counted and removed to separate containers, to determine their ability to complete development. If cysts appeared, they were filtered from the culture, dried, counted and resuspended in dilute sea water for hatchability determinations. When broods reached sexual maturity, the offspring were counted again and sexed.

Control data gave us reason to believe that quart jars are entirely adequate for survival records, but to make sure, crowding experiments were performed with much smaller 4-ounce jars. The experiments, repeated three times, involved a series of 2, 4, 8, 16, 32, 64, 128, 256 nauplii per 4 ounces of sea water. Results of crowding were evident when groups involved more than 32, but this took the form of repressed growth and delayed maturity rather than death. A feedback phenomenon, such as reported by Rose (1960) for fish and amphibia, is suggested.

During the summer all cultures were fed daily with yeast suspension, roughly at the rate of one drop per adult, added to the culture water. In addition they ate the volunteer algae present in the cultures. In fact pair matings and their offspring were maintained under constant illumination from banks of fluorescent tubes as customary in algae culture. The temperature on warm days reached 28° C. under such circumstances, at night and on cool days it fell off a degree or two. The temperature for mass cultures elsewhere in the room varied more than this during the growing period, averaging 25° C. but rising to 30° for afternoons when sun reached the cultures and falling to 20° on cool nights. This is much like the range in temperatures experienced by Bowen's (1962) cultures.

Pair mating tests were performed in sea water at the convenient specific gravity of 1.02 until 1961 when the comparisons were also made at higher and lower specific gravities within the range of adaptation found by earlier investigators (Jensen, 1918; Bond, 1932). Sea water was diluted to a specific gravity of 1.01 with distilled water. For high salinities NaCl was stirred into sea water to raise the specific gravity to 1.07 and 1.12. Adults typically survived transfer directly to 1.01 (lower) or 1.07 (higher) specific gravities, but rarely survived transfer to the 1.12 brine. Therefore gradual conditioning was attempted by daily additions of twelve successive equal doses of salt until a specific gravity of 1.12 was reached. However, only about 10% of the young adults used survived such conditioning.

RESULTS

Survival of cultures

Control cultures have been prolific and maintain themselves without difficulty. On the other hand, experimental cultures may be sparsely populated and those experiencing higher levels of radiation quickly trend to extinction. This results from reproductive failure rather than any other obvious influence on the treated adults. A summary of cultures begun and those which failed to survive is given by Table I.

In the P^{32} series, 3-L. cultures above 90 μc. have failed to survive. This limit was demonstrated for both the 1958 and 1959 series of experiments. The most persistent case was a sparse population in a 120-μc. jar which survived the 1959–60 overwintering but during the summer of 1960 did not expand successfully. Subcultures of 30-μc. experiments have not survived a repeat dose of 30 μc. of P^{32}. Furthermore, after 14–15 generations, the 1958 series of P^{32} cultures have entered a period of decline and seem to be on the verge of extinction. Cultures of the 1959 series which have gone through only nine generations appear to be in better condition.

In the Zn^{65} series *Artemia* cultures have survived only the lowest dose, 30 μc./3L. In the x-ray series persistent cultures have not been obtained from *Artemia* receiving more than 1000 r. In one culture from *Artemia* which had received 1000 r, nine generations have now elapsed. In 1960 the culture whose ancestors received 1000 r of x-ray appeared superior to the culture begun at the same time

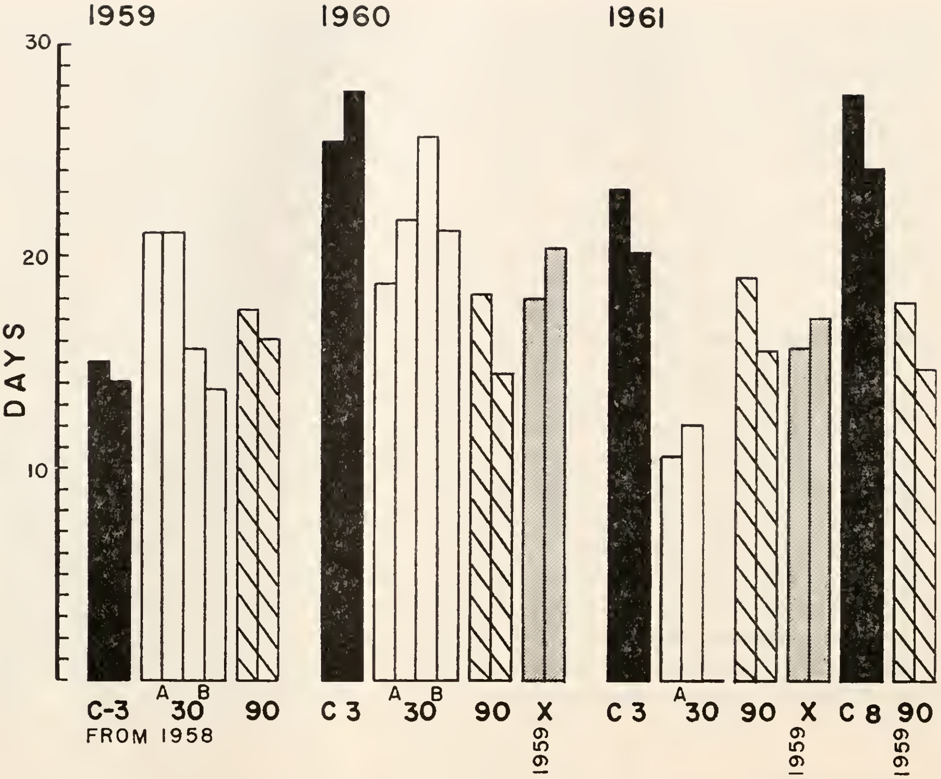

Figure 1. Survival of adult *Artemia* after transfer to quart jars for pair mating tests. The summer in which the data were obtained is identified by the year shown at the top; the cultures from which the animals were taken are designated along the bottom of the figure. C indicates control; X stands for x-rayed; 30 and 90 refer to the μc. of P^{32} added to respective 3-liter cultures at the start of the experiments. Female survival is given by the right bar of each pair.

incorporating 90 μc. of P^{32}. The x-ray culture provided adequate numbers of pairs for testing during a period when the P^{32} jar was too sparsely populated.

Duration of life

The average number of days between their transfer to quart jars and the death of members of mated pairs of *Artemia* is taken as a measure of adult life span (Fig. 1). Typical standard errors associated with these values range from 2.40 to 2.49 days for males and from 3.12 to 3.65 days for females. In 1959, experimental animals lived as long as or longer than the controls from culture #3. In subsequent years, individuals whose ancestors were subjected to radiation tended to die sooner than #3 controls. In 1961 an additional control, #8, was sampled. This has been maintained in exactly the same size and shape of jar as all experimental cultures. As shown, the adults withdrawn from #8 lived even longer than those from #3. Therefore, size and shape of container are ruled out as influences in poor life-span of experimental adults.

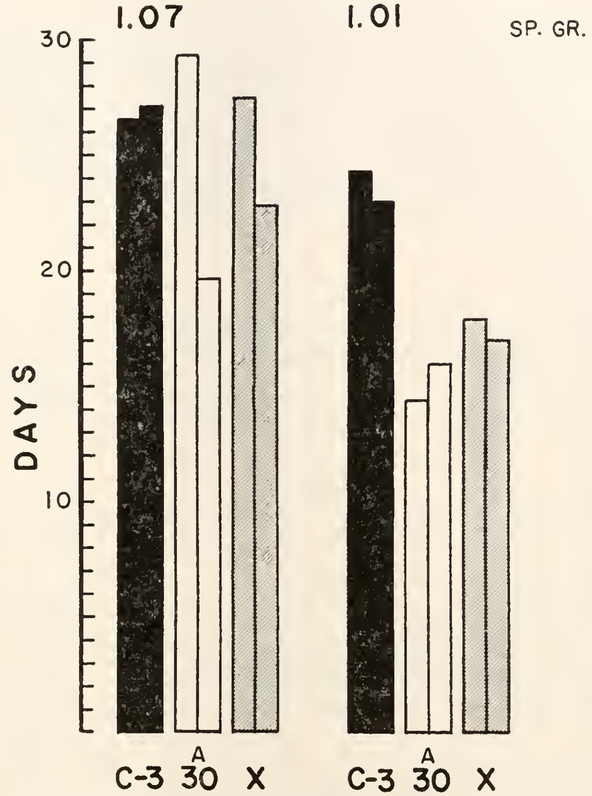

FIGURE 2. Survival of adult *Artemia* used for pair mating tests in sea water to which NaCl had been added (1.07 sp. gr.) and in diluted sea-water (1.01 sp. gr.). The respective culture supplying the animals is designated along the bottom: C-3 indicates control #3, A 30 refers to 30 μc. added to a 3-L. culture in 1958, X stands for the culture whose original parents were x-rayed in 1959. Female survival is given by the right bar of each pair.

Pair matings from the 1958 cultures receiving 90 μc. of P^{32} have given relatively consistent life span records in successive years. This is true also for the duplicate experiment begun in 1959. Furthermore, similar life spans have been obtained from the acute dose of 1000 r of x-rays. On the other hand, the cultures derived from a 30-μc. P^{32} treatment have varied over the years of study. Recently adult life span has proved brief in ordinary sea water. Additional results which are not included on the figure are for the 30-μc. Zn^{65} culture begun in 1960. Average survival of males was 18.6 ± 3.95, and 19.3 ± 2.28 days for females.

When brine of 1.07 specific gravity was used for tested pairs, adult life span was prolonged. The proper comparison is between the 1.07 results of Figure 2 and the 1961 results of Figure 1 for the same three cultures. The standard errors for Figure 2 values are within the range stated above for Figure 1 values.

In brine of higher specific gravity, 1.12, the two pairs of control adults which survived conditioning had lengthy spans of life: 31 and 36 days for males, and 28 and 24 days for females. P^{32} animals did not do as well. Four conditioned pairs averaged 22.5 and 13.2 days for males and females, respectively. From the x-ray population, the one pair conditioned died after only 11 (female) and 15 (male) days.

Figure 2 also presents survival of adults in dilute sea water of 1.01 specific gravity. These values are better but do not impressively exceed the 1961 sea-water results for the same culture (Fig. 1). It is impossible to run all tests simultaneously so some improvement might be due to increased experience of the assistant. However, in this case less variability might be expected. Instead, standard errors exceed 4 days, and for males of control #3 is a high 5.22 days.

Components of fitness

Life span is part of the story, but it is possible to examine the various aspects of reproductive failure more directly. The summaries in Tables II and III indicate whether mated pairs are likely to give rise to sexually mature offspring. Adaptive values epitomize the reproductive efficiency of a genotype in a certain environment.

TABLE II

Reproductive behavior of Artemia cultures as revealed by pair mating studies in sea water

Cultures treated at the date indicated	Number of broods per pair	Zygotes voided *		% Survived to adult	Mature adults per pair	Adaptive value
		Per pair	Per brood			
Results of pair matings in 1959						
Control #3	1.2	176.9	81.7	24.3	43.0	1.00
30 µc./3L. 1958						
"A"	0.9	31.6	27.4	24.4	7.7	0.18
"B"	0.7	85.1	52.7	26.0	22.1	0.51
90 µc./3L. 1958	0.6	31.8	39.8	20.0	6.4	0.15
Results of pair matings in 1960						
Control #3	2.6	387.4	149.0	43.1	156.97	1.00
"A"	1.6	165.9	103.7	27.6	45.79	0.29
"B"	2.6	381.9	146.9	31.5	120.30	0.77
90 µc. 1958	1.3	110.1	84.7	30.3	33.36	0.21
X- ray						
1000 r. 1959	1.0	106.5	106.5	31.2	33.32	0.21
Results of pair matings in 1961						
Control #3	2.2	179.5	81.6	50.3	90.3	1.00
Control #8	2.4	164.6	68.6	76.5	125.9	1.39
"A"	0.3	12.8	42.7	57.6	8.7	0.10
90 µc. 1958	0.2	6.1	30.5	0	0	0
X-ray 1959	0.5	19.93	39.9	14.5	2.9	0.03
90 µc./3L. 1959	0.3	7.08	28.3	32.7	2.3	0.03
Zn^{65}						
30 µc./3L. 1960	1.17	59.2	50.6	72.6	42.9	0.58

* Zygotes voided is used to refer to the number of nauplii and cysts deposited.

Table III

Reproductive behavior of Artemia cultures revealed in 1961 by pair mating studies in salt waters of higher and lower specific gravities than sea water

Cultures tested	Number of broods per pair	Zygotes voided *		% Survived to adult	Mature adults per pair	Adaptive value
		Per pair	Per brood			
Specific gravity 1.01						
Control #3	2.2	228.6	103.9	47.4	108.3	1.00
"A" 1958	0.8	20.5	24.6	26.4	5.4	0.05
X-ray 1959	1.1	51.2	46.6	62.8	32.2	0.30
Specific gravity 1.07						
Control #3	2.8	182.5	65.3	51.7	94.3	1.00
"A" 1958	1.9	107.3	60.4	61.9	66.4	0.70
X-ray 1959	2.1	85.4	41.0	69.0	58.9	0.62

* Number of nauplii and cysts deposited.

As defined (Dobzhansky, 1951) adaptive value is the relative capacity of carriers of a given genotype to transmit their genes to the gene pool of the following generations. On this basis we have taken our evidence of the average number of mature progeny produced per pair, assigned the unit value to control #3 and made the pertinent comparisons within each year.

Aspects in which a cultured population is deficient are revealed in these experiments where all voided zygotes and all adults developing therefrom are counted. Table II presents results for pair mating tests in three successive years, using sea water of 1.02 specific gravity. Table III presents data from 1961 experiments at lower and higher specific gravities.

Experimental populations have never approached the controls in their production of live offspring. "B" came closest in 1960 when as many broods were deposited and the number of zygotes per brood was only slightly lower. However, the curbing influence was revealed in fewer offspring surviving to adulthood. Subsequently decline of this culture has been so drastic that in 1961 it did not produce enough adults to allow pair mating tests. Adequate numbers of adults for the 1961 pair mating tests were provided by the survivor of the highest P^{32} level of 1958, 90 μc./3L., but Table II demonstrates poor performance in all the aspects considered. The culture which gave an adaptive value of zero in 1961 tests did not survive overwintering to 1962.

A small number of broods, a form of infecundity, has appeared for various experimental cultures tested by pair matings. At the same time, the decrease in the number of zygotes per brood may not be severe. Indeed, in 1961 "A" was producing considerably larger broods than it was in 1958 and survival to adulthood was better than for #3 control, yet relatively few adult offspring were obtained, chiefly because parents which were fertile produced only single broods. Earlier indications of the importance of fecundity came in 1959 when experimentals and controls differed only slightly in larval lethality, and in 1960 when there was little difference in larval lethality among various experimental groups. Finally in 1961, larval survival for "A" and for Zn^{65} pair mating tests surpassed that for the #3 control although neither adaptive value at all approaches the control value.

Table III demonstrates that *Artemia* react differently in their reproductive abilities when the specific gravity of the medium was made higher or lower than the convenient 1.02 of sea water. Particularly notable were the improvements in all reproductive aspects for "A" and the x-ray parents when brine of 1.07 specific gravity was used. Improvements, except in number of zygotes per brood, were also seen for control #3.

On the other hand, brine of 1.12 specific gravity did not improve the reproductive ability of *Artemia.* One or more broods were produced for the few pairs conditioned to this salty brine, but survival to adulthood was poor: 26.1% for controls, 9.3% for "A" and zero for larvae from x-ray parents. The number of offspring per brood was not good: 43, 26, and 14.5, respectively.

Hatchability of cysts

Although cysts occur regularly in the mass cultures which evaporate slowly during the winter, under the conditions of the pair mating tests, "winter eggs" appeared only occasionally. When obtained they formed the last brood or a part of it and contributed only a small fraction of the total zygotes voided. Table IV presents emergence or hatchability of the cysts, along with the number of mated pairs producing cysts. On the basis of the 1960 records it might seem that when females have a longer life span (Fig. 1) they are more likely to deposit an encysted brood, although this is not borne out by our subsequent experience. For example Control #8 in 1961 averaged 24+ days for female survival but deposited no cysts. Furthermore in brine of 1.07 specific gravity only 2 of 10 #3 control females deposited cysts, although the average survival for both sexes was 26–27 days.

If there is no sperm storage (Bowen, 1962) male life span could be a limiting factor on cyst deposition. However, from this standpoint the 1960 #3 control would drop back to 25 days for effective pair survival, which is not significantly different from the #8 1961 control and shorter than the 1961 #3 control values in brine (1.07 specific gravity).

TABLE IV

Cyst deposit and emergence from cysts

Source	1959		1960		1961	
	% emerged	No. laying cysts per 15 mated	% emerged	No. laying cysts per 20 mated	% emerged	No. laying cysts per 10 mated
Control #3	41.9	6	46.4	17	46.0	6
30 μc. P^{32} A	15.4	1	29.2	12		0
30 μc. P^{32} B	24.9	3	47.5	16		
90 μc. P^{32} 1958	18.1	1	58.5	6		0
X-ray			49.5	8	28.4	2
Zn^{65}					45.5	1

In spite of the inconsistent deposit of cysts by females maintained in isolated jars under continuous illumination, some insight is provided concerning survival of mass cultures. In 1960, the year when a large proportion of pairs produced cysts, tests of three out of four experimental cultures showed hatchability above the control values. On this basis hatchability does not seem to be a major influence upon survival of a culture.

Control values between 40 and 50% hatchable cysts are not unexpected in unselected samples. Unselected commercial samples of cysts from natural populations may give even lower hatchability. Flotation or some other method of eliminating deficient or empty cysts seems necessary to improve hatchability.

Sex ratio

A subtle difference between populous cultures and those which appear headed for extinction is revealed by summarizing the sex ratios of offspring reaching maturity. A vulnerability of females in treated populations suggests the segregation of deleterious induced recessives in the heterogametic sex (although the author is aware that the question of female heterogamety in *Artemia* is still controversial).

Table V demonstrates that the sex ratio tends to favor males when the parental pairs tested are drawn from cultures whose ancestors were irradiated. In eight out of ten sets of pair mating tests the control value for the particular year is exceeded. Chi square determinations provide significant values for 1960 "A" and

TABLE V

Sex ratios given as the ratio of males to females, and chi square values calculated from the original data

Origin of parents	1959	χ^2	1960	χ^2	1961	χ^2
Control #3	.82		.74		.91	
P^{32} 30 μc. A	1.25	2.069	.92	4.650*	1.31	3.450
P^{32} 30 μc. B	1.00	1.031	1.15	16.987**		
P^{32} 30 μc. 1958	.72	.124	.83	.907		
X-ray 1959			.68	.176	1.22	.860
Zn^{65} 30 μc.					1.02	1.380
Control #8					.95	.437
1.07 sp. gr. brine						
Control #3					.91	
A 1958					1.34	9.903**
X-ray 1959					.93	.006
1.01 sp. gr. dilute sea water						
Control #3					.88	
A 1958					1.40	3.262
X-ray					.87	.0004

* = significant.
** = highly significant.

"B" results as an indication that deviations are more than subtle in those cases. Note that in controls, more adult females were produced than males, while experimental cultures favor males.

At both higher and lower specific gravities the sex ratio favors males in tests of Culture A originally derived from ancestors exposed to 30 μc. of P^{32} in 1958. The chi square value for the 1.07 specific gravity test is highly significant. The culture from x-rayed ancestors, which at present is more prolific than "A", shows sex ratios not significantly different from those of #3 controls run at the same specific gravities.

Discussion

The fact that *Artemia* cultures derived from radioisotope- and x-ray-exposed ancestors are doing poorly may be viewed from several aspects although the problems of waste disposal, ecological disturbance and population genetics are interrelated.

In practice, where isotopic concentrations have been determined in the environs of the Hanford, Washington, nuclear plant, concentrations in the effluent water are much lower than the levels used for our experiments. Bustad (1960) reports 2×10^{-8} μc./cc. for P^{32} and 1×10^{-7} μc./cc. for Zn^{65}. These are activation products rather than discharged wastes. Another example is White Oak Lake which received effluents from Oak Ridge, Tennessee. Wastes here include fission products and transuranic elements, yet the average concentration in the water was estimated at 10^{-3} μc./cc., lower by at least a factor of ten than any of our experiments.

On the other hand, a document considered when the experiments were planned (NAS-NRC, 1959) gave a maximum permissible concentration of Zn^{65} in drinking water, 6×10^{-2} μc./cc. or 180 μc./3L., a level twice that at which *Artemia* can persist, and six times that which makes population survival difficult. The generalized concentration factor employed for invertebrates provided a more acceptable value of 1.2×10^{-4} μc./cc. as the permissible sea water concentration for Zn^{65}. In contrast, even without the invertebrate concentration factor, the MPC of P^{32} in drinking water (2×10^{-4} μc./cc.) was placed well below any level yet studied with *Artemia* populations. The recommendations were based on Handbook 52 of the National Bureau of Standards, now superseded by Handbook 69 in which permissible levels have been reduced for many isotopes.

In waters studied by ecologists it was the highest trophic levels which were damaged. Although species of fish were disappearing from White Oak Lake, and shortened life span and poor growth were reported for others, populations of aquatic insects were able to survive in spite of impressive concentration factors (Buchsbaum, 1958). Enormous doses of radiation may be necessary to destroy completely a primary trophic level such as an algal-protozoan community. No significant physiological or morphological damage to marine algae was demonstrated after the Bikini atomic tests (Blinks, 1952), although damage to the hereditary mechanism was not assessed. Doses such as those employed in the present experiment apparently seem in the range necessary to interfere with the primary consumers of the second trophic level, *Artemia* for example. Furthermore, the approach of the population geneticist is needed to reveal the nature and extent of the damage. Experimental *Artemia* showing no visible evidence in numbers or appearance of individuals for one or several generations, may carry hidden genetic damage responsible for subsequent decline to a dangerously small population.

Diptera have been the preferred material for such research, even for estimating genetic damage from the Caroline Islands atomic tests (Stone *et al.*, 1957; Stone and Wilson, 1958). Experimental procedures included population sampling by brother-sister matings. Reproductive performance, studied under laboratory conditions, revealed that direct irradiation and fallout damaged *Drosophila ananassae* populations severely. Many mutants and gene combinations interfered with development to adulthood, a difficulty demonstrated again in the present *Artemia* experiments. In spite of viability problems, the *Drosophila* populations have managed to return to normal reproductive performance, presumably through the operation of natural selection. The flies required from 26 to 161 generations to achieve reproductive recovery. Little more than half the lower number of generations has elapsed for the oldest *Artemia* culture. It will be interesting to see whether any of the irradiated *Artemia* populations can accomplish a recovery to normal levels of reproductive performance.

For *D. ananassae* no consistent relation of egg counts to genotype was detected (Stone *et al.*, 1957) although survival-extinction predictions for *D. melanogaster* are based in part upon fecundity (Wallace and Dobzhansky, 1959). Since the maximum number of possible offspring depends upon the number of functional eggs produced, there is a certain number of eggs required per female if the population is not to become extinct when exposed to a given amount of radiation. Fecundity as well as zygote viability is under genetic control and subject to irradiation damage, so that two dose-dependent aspects of survival are interrelated. With the exception of "B" in 1960, our experimental *Artemia* cultures have shown poor fecundity from the beginning. Possibly in a viviparous animal this matter is more serious than in an oviparous form. Insurmountable crises in development may occur which result in elimination of the zygote before deposit. Indeed, our category of "zygotes voided" may really reflect early embryo death and resorption as well as egg productivity. The cysts, which are often incorrectly called "eggs," are really embryos as far along as the blastula stage.

The price paid for the elimination of detrimental and lethal factors from a population is death of individuals, actual or potential. Our *Artemia* populations may now be paying this price. Controversy exists concerning (a) the retention of seemingly deleterious chromosomes for virtue of their characteristics in heterozygous individuals (Wallace, 1956), and (b) whether ambivalent mutants exist which impair fitness when homozygous but improve that of their heterozygous carriers (Wallace and Dobzhansky, 1959). High adaptive values for irradiated *Drosophila* populations have been reported (Wallace and King, 1951; Wallace, 1951), and the adaptive value for one acutely irradiated population even exceeded control values. In this case an x-ray dose of 1000 r was delivered to females and seven times that dose to males. In contrast to the *Drosophila* results, experimental cultures of *Artemia* whose ancestors received 1000 r. to both sexes are clearly inferior to control populations. Indeed, for experimental *Artemia*, none of the adaptive values approach the high values reported for *Drosophila*. However, here again a comparable number of generations has not elapsed. By 1956, Wallace's populations had been followed for 150 generations; by 1959, 200 generations had elapsed. In addition there are a number of other features, such as size of organism and irradiation in water vs. air, which complicate a comparison. Furthermore

Wallace's *Drosophila* populations involved a contrived genetic background, an intentional isogenicity not readily obtainable with other organisms. Also, from a cytological standpoint it may be significant that *Drosophila* has a small number of chromosomes, some of which are long, possibly an ideal situation for fixation of chromosomal polymorphism. In contrast, *Artemia* has a large number of short chromosomes.

Selection experiments clearly indicate the accumulation of genetic lethals in irradiated laboratory stocks of *Drosophila* (Muller, 1950), but Wallace argued that fitness of a population consisting mainly of heterozygous individuals may be excellent, provided the population is large enough so that segregation of detrimental homozygotes will not threaten its existence. Perhaps our populations of several hundred *Artemia* are dangerously small, but this reflects our decision to devote facilities and efforts to a number of cultures encompassing a range of treatments, rather than to a few enormous populations which might have been given treatments too low for sharply defined comparisons. Actually, results from populations of limited size may be especially pertinent for practical considerations in other organisms. Although seasonally dense populations of *Artemia* occur in some salterns, such cases may be exceptional in present day ecology. Field studies have shown that most of the species present in a locality are represented by only a few individuals (Williams, 1953).

Doubt has been cast on improvement resulting from irradiation through a neoclassical version of heterosis. If mutations increasing the viability of the heterozygote are not demonstrable under favorable conditions for their detection, they are not too helpful an explanation of conditions in natural and experimental populations (Muller and Falk, 1961). Only decreases in the average viability of an otherwise homozygous *Drosophila melanogaster* genotype were obtained for radiation-induced mutations in heterozygous and unselected conditions (Falk, 1961). Furthermore, in laboratory conditions no significant influences on heterozygote viability were demonstrable for *D. willistoni* lethals, whether natural or induced (da Cunha *et al.,* 1959). In plants, Stadler's (1932) pessimism about the damaging aspects of radiation-induced mutation is traditional, although for cultivated crops desirable traits may emerge from irradiated populations under the practice of artificial selection (Gustafsson, 1947; Sparrow and Singleton, 1953; Konzak, 1954; Gregory, 1956). Finally, to date, only detriment has been demonstrated for *Artemia* cultures descended from irradiated ancestors.

A notable point concerning *Artemia* biology has emerged from these studies. Increased life span and improved reproductive performance in brine (1.07 specific gravity) indicate favorable aspects in addition to a lack of predators (Lochhead, 1941) in the niche with which *Artemia* is associated. Other recent investigators feel it desirable to culture *Artemia* in water saltier than sea water. Bowen's (1962) standard procedure is to add NaCl as we have done. After trials with different concentrations, Goldschmidt (1952) adopted a standard specific gravity of 1.04 obtained by evaporation.

Summary

1. Results are presented for four years of study on the survival of *Artemia* cultures when ancestors have been exposed to a series of doses of either radioisotopes

or x-rays. Cultures were begun by transferring 10 pairs of adults from a control culture to a 3-liter jar of sea water. Ordinarily, within a generation this gives rise to a culture of several hundred animals.

2. Three-liter cultures did not persist if more than 90 μc. of P^{32} or more than 30 μc. of Zn^{65} have been added. Subcultures of 30 μc. of P^{32} per three liters did not survive a second dose of 30 μc./3L. Also, cultures failed if 2000 r or more of x-rays were delivered to the 10 pairs of adults used to institute the culture.

3. The treatments investigated had no obvious effect upon the original adults. Decline and extinction of the cultures occurred at the first or subsequent generations of offspring.

4. In order to assess reproductive failure, pairs when sexually mature were transferred from the 3-L. cultures to quart jars. All zygotes voided were counted and hatchability was determined for any cysts deposited. Each brood was transferred to a separate container. Progeny surviving to adulthood were counted again and sexed.

5. (a) Decrease both in number of zygotes voided and in survival to adulthood contributed to low adaptive values for experimental organisms.

(b) The sex ratio among offspring tends to favor females in control and males in experimental material.

6. Routinely the convenient specific gravity of 1.02 has been used for pair matings and spring reactivation of mass cultures. In 1961 pair mating tests were run in dilute sea water of 1.01 specific gravity and in sea water to which NaCl had been added to reach a specific gravity of 1.07. Both life span and reproductive behavior were improved in brine of 1.07 specific gravity. However, attempts to condition adults to saltier brine of 1.12 specific gravity were rarely successful and reproductive performance of the few shrimp conditioned was poor. Evidently there is an optimum brine range for *Artemia*, involving more fundamental biological aspects than previously reported.

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NEUROSECRETION AND CRUSTACEAN RETINAL PIGMENT HORMONE: ASSAY AND PROPERTIES OF THE LIGHT-ADAPTING HORMONE[1]

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Within the past three decades, hormones from the X-organ-sinus-gland complex of the crustacean eyestalk have been shown to participate in a variety of physiological systems: color change, photomechanical movements of retinal pigments, hyperglycemia under stressing conditions, molt, regeneration, and ovarian growth. Such physiological effects have been reviewed in detail by a number of contributors to a study of the physiology of Crustacea (Bliss, 1960; Charniaux-Cotton, 1960; Florkin, 1960; Kleinholz, 1961; Passano, 1960; and Welsh, 1961). It is apparent from these reviews that the physical and chemical properties of the reported active principles are not well known, although such information would be valuable in resolving the number of different hormones responsible for the variety of physiological effects obtained with crude extracts of eyestalks. The erythrophore-concentrating hormone has been the only one reported as a purified preparation (Edman, Fänge and Östlund, 1958) but no thorough tests have been made either of its chemical or physiological homogeneity; this preparation shows no activity in light-adapting distal retinal pigment (Kleinholz, 1958; Kleinholz *et al.*, 1962). Knowledge of the properties of these eyestalk hormones would be helpful not only in indicating their chemical nature but also in their separation, purification and subsequent chemical identification.

Such anticipated separation and purification attempts will require assay methods for each of the active principles being investigated. Abramowitz (1937) has described a biological assay of chromatophore hormone based on the melanophore of *Uca,* while Sandeen (1950) and Fingerman (1956) have measured erythrophore responses to hormone by methods that might be developed into an assay procedure. It has been shown that the distal retinal pigment of *Palaemon adspersus* (Kleinholz and Knowles, 1938) and of *Palaemonetes vulgaris* (Sandeen and Brown, 1952) assumes positions intermediate between the extremes of light- and of dark-adaptation related to intensity of illumination. Since a range of concentrations of injected eyestalk extract produces a similar graded response of the distal retinal pigment in *Palaemon* (Kleinholz, 1938), it is postulated that normal photomechanical movement of these effectors may be regulated by the amount of hormone liberated into the circulatory system, and that an assay for this hormone could be devised on the basis of these observations.

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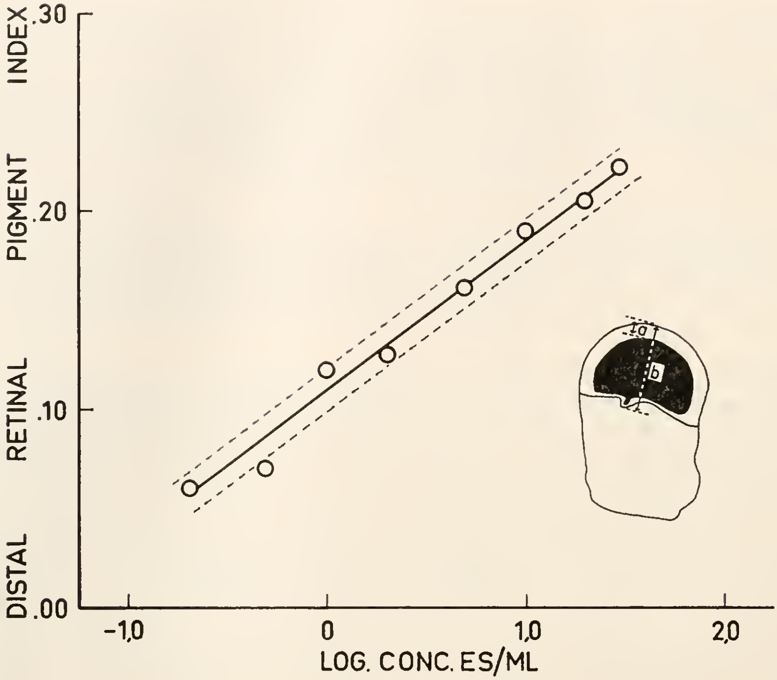
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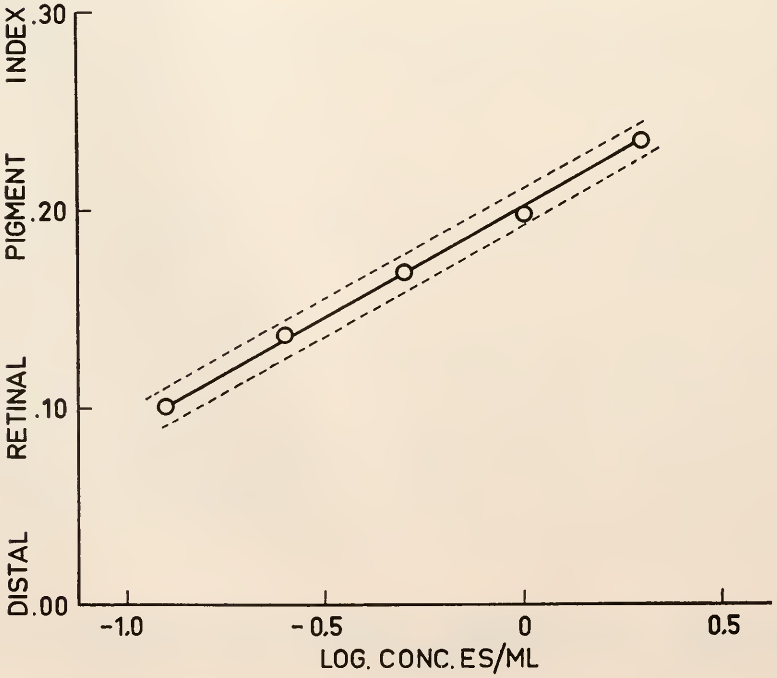
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The present report describes dosage-response relations for the light-adapting retinal pigment hormone, and several properties of this principle. Both kinds of examination were undertaken as necessary preliminaries to a systematic attempt at isolating the hormone in pure form.

Materials and Methods

The decapod crustaceans, *Libinia emarginata* Leach (males, weighing 500–550 grams), *Palaemonetes vulgaris* Say (not selected by sex but including a large proportion of ovigerous females, 35–40 mm. rostrum-telson length) and *Carcinus maenas* Linnaeus (males, approximately 5 cm. in maximum carapace width) were donor species whose eyestalks were used to construct dosage-response curves for the distal retinal pigment. *Palaemonetes vulgaris* [2] was the test animal for the first two donor species and *Palaemon adspersus* [2] for the third. Eyestalks from the light-adapted donor species were triturated with a small amount of reagent-grade sand and were extracted with measured amounts of solvent (distilled water for *Palaemonetes* eyestalks, sea water for the others). The tissue suspensions were centrifuged and the supernatants injected into test animals within an hour after the extractions were begun.

Test and control animals, isolated in individual containers, were dark-adapted for 3–10 hours before injection. At timed intervals, 0.05 ml. of the prepared extract was injected into a test animal by the dim light from a red lamp; uninjected control animals were exposed to the same light for comparable periods. Forty-five minutes after injection (Welsh, 1930; Kleinholz, 1936, 1938), response of the distal retinal pigment cells was measured. The slight modification of the Sandeen and Brown (1952) method of recording the response as a "distal retinal pigment index" (DRPI) has been described (Kleinholz *et al.,* 1962). Briefly, the ratio of two measurements (distance from the cornea to the distal margin of the distal retinal pigment, and distance from the cornea to the proximal margin of the dorsal pigment spot shown in Figure 1) furnishes the DRPI. The dosage-response curves are based on a minimum of 10 injected test animals (*i.e.,* 20 retinas) for each concentration of extract.

Stability of distal retinal pigment light-adapting hormone (DRPLH) to drying, heating and freezing was examined. An extract of 10 eyestalks of *Libinia* in 1 ml. distilled water was heated for 2 minutes at 100° C. and centrifuged. Three 100-μl. aliquots of the supernatant were applied to strips of filter paper and dried in a stream of warm air; the paper strips were then stored under vacuum at 20° C. After 1, 6, and 20 days of storage one of the paper strips was eluted for 2 hours with 0.5 ml. distilled water. The eluates, equivalent to concentrations of 2 eyestalks per 1 ml., were tested for activity by injection into dark-adapted *Palaemonetes.*

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[2] The systematic nomenclature of these crustaceans has recently undergone revision. The new names were also used in the first report in this series, Kleinholz *et al.* (1962).

FIGURE 1. Regression of distal retinal pigment index (response) on logarithm of eyestalk concentration of injected extracts. The upper figure is for *Palaemonetes* eyestalk extract, with the standard error of the estimate shown in broken lines. The inset drawing of an eyestalk shows the measurements made for calculating the DRPI. The lower figure is for *Libinia* eyestalk extract. The test species for both figures is *P. vulgaris.*

The effect of heat on activity of retinal pigment hormone was examined by comparing DRPI responses produced by extract of eyestalks dried 2 hours at 110° C. with responses given by extracts prepared from fresh, unheated eyestalks of the same donors. Ablated eyestalks, one from each of 15 light-adapted *Palaemonetes,* were placed in the drying oven. The remaining eyestalks, removed immediately thereafter, were ground and extracted in 1.5 ml. distilled water, centrifuged, and the supernatant injected into dark-adapted *Palaemonetes.* The oven-dried eyestalks were then similarly extracted and tested.

Hormone activity in frozen-dried eyestalks was compared with that in oven-dried eyestalks. One eyestalk from each of 20 *Palaemonetes* was collected and frozen in a small boat of aluminum foil kept on solid CO_2, while the second eyestalks from these donors were dried at 115° C. The frozen eyestalks were lyophilized, after which both sets of eyestalks were stored at room temperature in a vacuum desiccator over anhydrous $CaSO_4$. On the following day extracts were prepared in concentrations of 20 eyestalks per 1 ml. and were tested.

Solubility of retinal pigment hormone in ethanol and in acetone was determined. Sets of eyestalks from light-adapted *Palaemonetes* were dried for 3–7 hours at 115° C. and stored in a vacuum desiccator over anhydrous $CaSO_4$ until used. One set of dried eyestalks was homogenized and extracted with distilled water; the second set of contralateral eyestalks from the same donors was extracted with 100% ethanol that had been dried for the preceding 24 hours over CaO. After centrifugation, each residue was washed with its appropriate solvent. The combined ethanol supernatants were evaporated to dryness at 115° C., and the residue dissolved in distilled water. The homogenized tissue remaining after ethanol extraction was then extracted with distilled water. The final extracts thus represented the original control aqueous extract, the ethanol-soluble extract, and the ethanol-insoluble extract, all now in aqueous solutions whose concentrations were adjusted to 10 eyestalks per 1 ml. Activity of the 100% ethanol fraction was measured on two successive days, the extracts being stored at −20° C. in the interim. The same procedure was used to extract dried eyestalks with 95% ethanol, and with acetone containing 1% glacial acetic acid.

Dialyzability of retinal pigment hormone was tested with Visking cellophane tubing. Distilled water extracts of *Libinia* eyestalks were heated for 2 minutes at 100° C., centrifuged, and 1 ml. of the supernatant, equivalent to 10 eyestalks, was dialyzed at 10° C. against 1 ml. of distilled water. Samples of the dialysate were injected into test *Palaemonetes* after 3 and 24 hours of dialysis; the contents of the cellophane bag were also tested for activity after 24 hours of dialysis.

Gradual inactivation of retinal pigment hormone, found to occur in freshly prepared extracts allowed to remain at room temperature, was suspected of being mediated by tissue enzymes, and the rate of inactivation was examined from this aspect. An extract of 10 eyestalks of *Palaemonetes* in 1 ml. of filtered sea water was prepared; immediately after centrifugation a portion of the supernatant was tested at "zero" hours by injection into 5 dark-adapted *Palaemonetes.* The rest of the supernatant solution, kept at about 25° C., was tested for activity at intervals thereafter of 3, 6, 10.5 and 12 hours. Two modifications in procedure were made to minimize the possible role of micro-organisms in the sea water. In one modification an extract of 40 *Palaemonetes* eyestalks in 4 ml. of *distilled* water was divided into two parts, one of which was heated at 100° C. for 1 minute. The extracts were then centrifuged and both supernatants tested for activity at "zero" hours. The two solutions, kept at 25–27° C., were tested again 12 and 24 hours afterwards. In the second modification, the supernatant of a centrifuged extract containing 40 *Palaemonetes* eyestalks in 2 ml. of distilled water was divided into two equal portions. One sample was diluted with an equal volume of distilled water, while to the second was added an equal volume of antibiotic solution (10 mg. Parke-Davis crystalline Penicillin G-potassium and 10 mg. Squibb Mycostatin in 50 ml. distilled water). The two extracts and a control consisting of the antibiotic solution were injected into groups of 5 test *Palaemonetes* at "zero" hours; a second test of the two eyestalk extracts was made 11 hours later. The amount of inactivation of the DRPLH was calculated from:

$$100\% - \left(\frac{\mathrm{DRPI}_t - \mathrm{DRPI}_c}{\mathrm{DRPI}_o - \mathrm{DRPI}_c} \times 100 \right) = \%\ \text{inactivation}$$

where DRPI_t = the average DRPI produced by unheated extract at the various intervals after its preparation; DRPI_o = the average DRPI produced either by extract tested at "zero" hours or by heated extract; DRPI_c = the average DRPI, 0.050, found for a large series of dark-adapted, uninjected control *Palaemonetes*.

The optimum pH for this inactivation was determined and distribution of the enzyme in a variety of tissues was examined. A stock enzyme solution was prepared by homogenizing 200 *Palaemonetes* eyestalks in an ice bath and by extracting the homogenate with small amounts of iced 1% NaCl solution. The supernatant, after centrifugation, was dialyzed for 20–24 hours at 3° C. against three changes of 1.5 liters of 1% NaCl, to remove retinal pigment hormone, and was made to a volume of 2 ml. The retinal pigment hormone substrate was a partially-purified preparation containing the equivalent of 200 eyestalks of *Palaemonetes* per 1 ml. Both preparations were stored at −20° C., samples being removed as needed from the thawed solutions. For subsequent tests, 0.3 ml. of distilled water and 0.1 ml. of the enzyme preparation were added to each of two centrifuge tubes, one of the tubes being heated for 2 minutes at 100° C. to denature the enzyme and serve as a control. Appropriate buffer, 1.5 ml., and 0.1 ml. of the retinal pigment hormone preparation were then added to each tube and the mixtures incubated for 6 hours at 38° C. After centrifugation and checking the pH of the supernatants, hormonal activity was tested, the supernatant containing the undenatured enzyme being injected first, generally within 15 minutes after removal from the incubator. The amount of inactivation of the DRPLH was calculated as described above. The buffers used were: 0.1 *M* succinate, 0.2 *M* borate, and 0.2 *M* Tris maleate, to provide a series of pH concentrations ranging from 5.1 to 9.1.

A number of tissues other than *Palaemonetes* eyestalks were examined for the presence of this hormone-inactivating enzyme. Preparation of the enzyme extract from these tissues was made as described above for eyestalks; quantitative details are summarized in Table II. A volume of tissue brei was placed in each of two tubes, one of which was heated to denature the enzyme. To both tubes were added 1.5 ml. of 0.2 *M* Tris maleate buffer at pH 7.4 and 0.1 ml. of retinal pigment hormone solution. After incubation for 6 hours at 38° C. the mixtures were centrifuged, and the supernatant tested for activity.

The effect of proteolytic enzymes on retinal pigment hormone activity was tested with several preparations. Aqueous extracts of eyestalks in known concentration were heated briefly in a boiling water bath to coagulate eyestalk debris. The supernatant, after centrifugation, was divided into two equal portions, enzyme being added to one while the other served as a control. Incubation at 35–38° C. was for varying periods (Table III), after which both mixtures were immersed in a 100° C. water bath for 1–2 minutes, centrifuged, and the activity of the supernatants tested. At Naples, extract was prepared from eyestalks of *Palaemon serratus* and tested on *Palaemon xiphias;* at Woods Hole, donor and test species were *Palaemonetes vulgaris.* Salt-free crystalline trypsin and chymotrypsin (Worthington Biochemical Co.) and a crystalline chymotrypsin (Armour and Co.) containing 50% ammonium sulfate were the enzymes used.

OBSERVATIONS

1. *Dosage-response relations*

Eyestalk extracts of *Palaemonetes* give the following average DRPI values and the calculated standard deviations when injected into dark-adapted *Palaemonetes* in

TABLE I

Properties of the light-adapting distal retinal pigment hormone. Activity tests were made on dark-adapted Palaemonetes vulgaris, *as described in the text.* DRPI, *average distal retinal pigment index for a test group and the standard deviation;* ES, *eyestalks.*

Eyestalk extract		Results	
Donor species and concentration	Treatment	Experimental DRPI ± S.D.	Control DRPI ± S.D.
P. vulgaris: 10 ES/ml.	Oven-dried ES vs. control fresh ES	0.215 ± 0.01	0.200 ± 0.02
P. vulgaris: 5 ES/ml.	Oven-dried ES vs. control fresh ES	0.143 ± 0.04	0.181 ± 0.03
P. vulgaris: 20 ES/ml.	Lyophil. ES vs. oven-dried control ES	0.222 ± 0.01	0.229 ± 0.01
P. vulgaris: 10 ES/ml.	100%-EtOH-sol. extract vs. H_2O-extract of oven-dried ES	0.090 ± 0.01	0.227 ± 0.01
	100%-EtOH-insol. extract vs. H_2O-extract of oven-dried ES	0.214 ± 0.01	0.227 ± 0.01
P. vulgaris: 10 ES/ml.	95%-EtOH-sol. extract vs. H_2O-extract of oven-dried ES	0.161 ± 0.04	0.182 ± 0.05
P. vulgaris: 10 ES/ml.	Acetone-HAc-sol. extract vs. H_2O-extract of oven-dried ES	0.049 ± 0.01	0.225 ± 0.01
	Acetone-HAc-insol. extract vs. H_2O-extract of oven-dried ES	0.215 ± 0.01	0.225 ± 0.01
L. emarginata: pre-dialysis 10 ES/ml.	Dialysate after 3 hrs.	0.113 ± 0.02	
	Dialysate after 24 hrs.	0.196 ± 0.06	
	Dialysate after 22 hrs.	0.215 ± 0.02	

TABLE I—(*Continued*)

Eyestalk extract		Results	
Donor species and concentration	Treatment	Experimental DRPI ± S.D.	Control DRPI ± S.D.
P. vulgaris: 10 ES/ml.	Inactivation at 25° C.:		
	0 hrs.		0.189 ± 0.04
	3 hrs.	0.144 ± 0.04	
	6 hrs.	0.114 ± 0.03	
	10.5 hrs.	0.084 ± 0.02	
	12 hrs.	0.049 ± 0.01	
P. vulgaris: 10 ES/ml.	Inactivation of unheated extract vs. control heated extract:		
	0 hrs.	0.208 ± 0.02	0.216 ± 0.01
	12 hrs.	0.118 ± 0.04	0.215 ± 0.01
	24 hrs.	0.100 ± 0.02	0.200 ± 0.02
P. vulgaris: 10 ES/ml.	Inactivation of extract + antibiotic vs. control without antibiotic		
	0 hrs.	0.208 ± 0.02	0.199 ± 0.01
	11 hrs.	0.077 ± 0.01	0.062 ± 0.02
P. vulgaris: ca. 20 ES/ml.	pH optimum of inactivation; unheated extract vs. extract with enzyme denatured:		
	pH 5.1	0.171 ± 0.03	0.178 ± 0.03
	pH 6.0	0.179 ± 0.02	0.226 ± 0.01
	pH 6.7	0.133 ± 0.04	0.195 ± 0.02
	pH 7.3	0.104 ± 0.03	0.205 ± 0.02
	pH 8.0	0.131 ± 0.03	0.222 ± 0.02
	pH 9.1	0.155 ± 0.04	0.161 ± 0.04

the concentrations shown: 30 eyestalks per ml. = 0.223 ± 0.018; 20 eyestalks per ml. = 0.205 ± 0.019; 10 eyestalks per ml. = 0.190 ± 0.031; 5 eyestalks per ml. = 0.161 ± 0.029; 2 eyestalks per ml. = 0.127 ± 0.036; 1 eyestalk per ml. = 0.120 ± 0.022; 0.5 eyestalk per ml. = 0.070 ± 0.015; 0.2 eyestalk per ml. = 0.061 ± 0.019. The relation between these data is linear when concentration of injected eyestalk extract is plotted on a logarithmic scale (Fig. 1). The equation for this relation is: $Y = 0.108 + 0.077 \log X$, where Y is the average DRPI for 10 test animals, and X is the concentration of the injected extract, within the limits of the upper and lower thresholds. The standard error of the estimate is ±0.008 DRPI.

Extracts of *Libinia* eyestalks tested on *Palaemonetes* result in the following average DRPI values and their standard deviations: 2 eyestalks per ml. = 0.235 ± 0.015; 1 eyestalk per ml. = 0.197 ± 0.039; 0.5 eyestalk per ml. = 0.168 ± 0.053; 0.25 eyestalk per ml. = 0.137 ± 0.022; 0.125 eyestalk per ml. = 0.101 ± 0.011. The upper threshold concentration is about 2 eyestalks per ml., because the next higher concentration tested, 4 eyestalks per ml., gives a DRPI of 0.237 ± 0.019. The linear relation resulting from a plot of the average response against the logarithm of concentration has for its equation: $Y = 0.200 + 0.107 \log X$, with the standard error of the estimate being ±0.007 DRPI.

TABLE II

Inactivation of retinal pigment hormone by tissue brei. DRPI$_u$, *average distal retinal pigment index of unheated extract and its standard deviation;* DRPI$_h$, *average distal retinal pigment index of heated control extract and the standard deviation.*

Species	Tissue used	Saline vol. used for making tissue brei	Brei vol. in incubated mixtures	DRPI$_u$	DRPI$_h$	Inactivation
Libinia emarginata	Heart; wet wt. = 0.6 gm.	2 ml.	0.2 ml.	0.140 ± 0.04	0.205 ± 0.03	41%
	Vas deferens from 2 males	2 ml.	0.4 ml.	0.050 ± 0.01	0.212 ± 0.03	100%
	Thoracic muscle = ca. 1 gm. wet wt.	4 ml.	0.4 ml.	0.061 ± 0.01	0.195 ± 0.03	92%
	Hypodermis from 2 carapaces	2.5 ml.	0.4 ml.	0.055 ± 0.02	0.232 ± 0.02	97%
	Blood; 3 ml.	0 ml.	0.4 ml.	0.213 ± 0.03	0.215 ± 0.01	1%
Palaemonetes vulgaris	Ventral nerve cord; 35 animals	1 ml.	0.4 ml.	0.093 ± 0.02	0.197 ± 0.03	72%
Pandalus borealis	50 eyestalks, = ca. 500 mg. dry wt.	2.5 ml.	0.2 ml.	0.151 ± 0.03	0.223 ± 0.02	42%
Mercenaria mercenaria	Adductor muscle, wet wt. = 2 gm.	4 ml.	0.4 ml.	0.059 ± 0.01	0.218 ± 0.03	95%

Similar tests with extracts of *Carcinus* eyestalks on *P. adspersus* as test animal yield the following DRPI responses: 15 eyestalks per ml. = 0.188 ± 0.025; 10 eyestalks per ml. = 0.196 ± 0.029; 5 eyestalks per ml. = 0.190 ± 0.031; 2.5 eyestalks per ml. = 0.178 ± 0.027; 1 eyestalk per ml. = 0.134 ± 0.015; 0.5 eyestalk per ml. = 0.129 ± 0.020; 0.2 eyestalk per ml. = 0.048 ± 0.012; 0.1 eyestalk per ml. = 0.035 ± 0.004. The upper threshold concentration seems to be about 5 eyestalks per ml. If these responses are plotted as a function of the logarithm of concentration of the injected extract the equation for the resulting linear relation is: $Y = 0.134 + 0.096 \log X$, with the standard error of the estimate being ±0.015 DRPI.

2. *Stability, solubility and dialyzability of the hormone*

Samples of aqueous extract of *Libinia* eyestalks, dried on filter paper strips and stored in vacuum, retain most of their activity. This is shown by average responses of 0.210 ± 0.02; 0.150 ± 0.04; and 0.170 ± 0.02 obtained when eluates from such paper strips made 1, 6, and 20 days, respectively, after storage are tested by injection. The concentration of the eluates (on the assumption that complete elution of hormone had occurred) was 2 eyestalks per ml.; the responses can be compared with the dosage-response curve for *Libinia* in Figure 1. A better controlled examination of the effects of drying on stability of retinal pigment hormone is shown in the next group of experiments. Extracts prepared from oven-dried *Palaemonetes* eyestalks give responses only slightly different from those produced by con-

TABLE III

Effect of proteolytic enzymes on activity of retinal pigment hormone. Prepared extracts, after heat treatment, were divided into two portions, enzyme being added to one and the other serving as control. The animals used were: P.s., Palaemon serratus; P.v., Palaemonetes vulgaris; P.x., Palaemon xiphias. *The crystalline enzymes used were:* T, *trypsin, and* C, *chymotrypsin; the designation in parentheses indicates the commercial source given in "Methods." Results are shown as* DRPI, *average distal retinal pigment index with the standard deviation, and the percentage inactivation, calculated as described.*

Eyestalk extract		Enzyme treatment			Results			
Donor	Conc.	Enzyme	Conc.	Incubation	Test species	DRPI ± S.D.		Inactivation
						Enzyme	Control	
P.v.	10 ES/ml.	T (WBC)	10 mg./ml.	12 hrs. at 35° C.	P.v.	0.136 ± 0.03	0.207 ± 0.02	45%
P.v.	10 ES/ml.	T (WBC)	5 mg./ml.	11 hrs. at 35° C.	P.v.	0.122 ± 0.03	0.193 ± 0.01	50%
P.s.	3 ES/ml.	C (ARM)	5 mg./ml.	19 hrs. at 37.5° C.	P.x.	0.097 ± 0.02	0.200 ± 0.01	70%
P.v.	10 ES/ml.	C (WBC)	8 mg./ml.	4 hrs. at 37.5° C.	P.v.	0.098 ± 0.02	0.204 ± 0.03	69%
P.v.	10 ES/ml.	C (WBC)	8 mg./ml.	13 hrs. at 37° C.	P.v.	0.075 ± 0.01	0.208 ± 0.01	84%

trol extracts of fresh eyestalks; similarly, extracts of lyophilized eyestalks result in test indices much like those obtained with oven-dried eyestalks (Table I).

Solubility studies, with precautions taken to avoid moisture in the solvents and in the eyestalk tissue, show little or no activity extracted by 100% ethanol or by acetone containing 1% glacial acetic acid. On the other hand, 95% ethanol does extract active material from oven-dried eyestalks. Some loss in activity occurred when the 100%-ethanol series (the ethanol-soluble, the ethanol-insoluble and the control aqueous extract) was thawed and tested after storage at −20° C.; the respective DRPI were 0.071 ± 0.01, 0.123 ± 0.04, and 0.188 ± 0.02, and an insoluble residue was present in each thawed preparation.

The hormone readily passes through a cellophane membrane, three hours of dialysis being sufficient to indicate the presence of activity in the dialysate (Table I). After 24 hours of dialysis, the tested dialysate produced a maximum response (compare with the dosage-response curve for *Libinia* in Figure 1).

3. *Enzymatic inactivation*

Spontaneous inactivation of retinal pigment hormone occurs regularly in extracts of fresh eyestalks, although the rate and degree of inactivation may be variable. Inactivation in one such experiment is shown in Table I, where the average DRPI, 0.189, of the freshly prepared eyestalk extract at 0 hours declines progressively until practically no activity remains 12 hours afterward. The rate of such inactivation is shown in Figure 2, with the percentage of inactivation being calculated as described in the section on methods.

A possible enzymatic basis for this inactivation is shown by tests with identical extracts, one of which is heated at 100° C. for 1 minute and serves as control for the unheated extract (Table I). The unheated extract results in an average DRPI of 0.208 at 0 hours and an average DRPI of 0.100 after 24 hours at 25–27° C., while the average activity values obtained with the heated control extract are not appreciably changed under these conditions. Eyestalk extracts of *Palaemon serratus* (5 per ml.) were tested in like manner on dark-adapted *Palaemon xiphias*. The extracts were divided into two portions, one being heated for 2 minutes at 100° C., and were then incubated at 37° C. for 15 hours. The average DRPI obtained with the unheated extract is 0.104 ± 0.03 (21 test animals), while that from heated extract is 0.193 ± 0.02 (17 test animals). The average DRPI for 26 dark-adapted, uninjected control *P. xiphias* is 0.052. The calculated percentages of inactivation are 68% for the *Palaemonetes* test and 63% for the *Palaemon* test, although incubation temperatures and experimental periods were not identical in the two cases.

Addition of antibiotic compounds to eyestalk extracts before incubation does not prevent loss of hormone activity. A curve constructed from the data in Table I over the range pH 5.1–9.1 shows the optimum for this inactivating enzyme to be pH 7.5. A summary of results from examination of a variety of tissues (Table II) shows that this enzyme is present in all tissues tested except blood.

Incubation of eyestalk extracts for different periods and with varying concentrations of trypsin or of chymotrypsin was made in a number of experiments, five of which are summarized in Table III. Nearly 50% of the activity originally present is inactivated by trypsin, while chymotrypsin brings about between 70–85% inactivation.

Discussion

The construction of dosage-response curves for the light-adapting distal retinal pigment hormone makes available a quantitative biological assay method for this hormone. The accuracy with which such assay can be made, however, will probably depend upon standardization of the procedure in the individual laboratory. The average DRPI values we obtain with control extracts of eyestalks from *Palaemonetes* and from *Libinia* show good agreement with those read from the dosage-response curves. Our suggested assay procedure is to obtain by serial dilution of the "unknown" the concentration producing an average DRPI slightly below the upper threshold response of the test animals. The average DRPI for this dilution and those obtained with two additional dilutions below this upper threshold concentration can then be substituted in the equation for the standard dosage-response curve to find their equivalent concentrations. Calculation of the average concentration of eyestalks in the original extract readily follows. It is evident from the examples reported here that the equations for such standard curves may vary with the species of the eyestalk donor and of the test animal, and it will therefore be necessary to construct such a standard curve for the particular species used.

The interconvertibility of such information from one laboratory to that from another would be aided by defining a physiological unit of hormone activity. For the present this can be done with data resulting from tests with *Palaemonetes* reported here. We therefore define the *Palamontes unit* for distal retinal pigment hormone as that concentration of eyestalks which, when injected into a minimum of 10 dark-adapted *Palaemonetes vulgaris*, measuring 35–40 mm. from rostrum to telson, yield an average DRPI of 0.150, this point being selected because it is about mid-way between the upper and lower threshold concentrations on the standard dosage-response curve. By this definition, 1 *Palaemonetes unit* is contained in *Palaemonetes* extracts having a concentration of 3.5 eyestalks per 1.0 ml. or in

Libinia extracts having a concentration of 0.34 eyestalks per 1.0 ml. After retinal pigment hormone has been isolated in pure form, dosage-response relations of whatever species were being used could be compared with the homogeneous preparation as a reference.

The stability and solubility properties described above show that eyestalks retain retinal pigment hormone activity after being oven-dried or lyophilized. This, and Carlson's (1936) report of chromatophorotropic activity in eyestalks dried and stored over a long period, have been useful in collecting and preparing quantities of eyestalk material for purification. We initially observed some loss in activity, accompanied by the formation of a precipitate, in fractionated eyestalk extracts thawed after storage at −20° C., and have therefore avoided repeated freezing and thawing of such preparations.

The *in vitro* inactivation by tissue extracts and by proteolytic enzymes point out additional interesting features of retinal pigment hormone. The variety of tissue extracts which inactivate the hormone, a pH optimum of about 7.5 for such inactivation, and the fact that the ability to destroy hormonal activity is thermolabile indicate a widely-occurring enzyme or group of enzymes. Whether such an enzyme system has an *in vivo* role in degrading hormone in the normal physiology of the retinal effectors is not known. Similar inactivation of chromatophorotropic hormone, first reported by Carstam (1951) for epidermis and later by Pérez-González (1957) and by Stephens and Green (1958) for a number of other

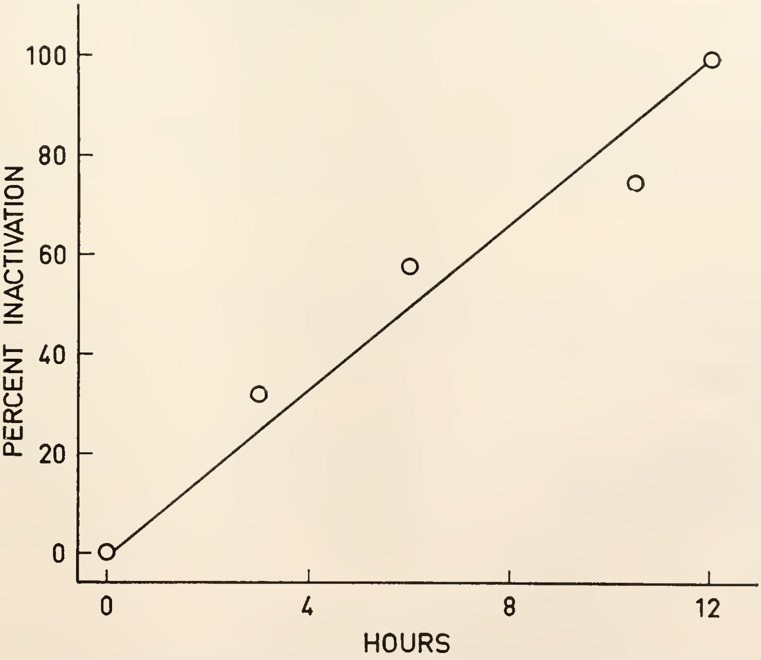

Figure 2. Rate of inactivation of distal retinal pigment hormone by enzyme in an eyestalk extract which was allowed to remain at room temperature for 12 hours. Extract was prepared from eyestalks of *Palaemonetes*.

crustacean tissues, may also explain apparent differences in hormone activity reported between boiled and unboiled eyestalk extracts.

The reduction by trypsin and by chymotrypsin of retinal pigment hormone activity described here was also confirmed by Fingerman and Mobberly (1960), after personal communication to them of our results. They too obtain partial loss of hormone activity in their trypsin-treated preparations. We observe a greater amount of inactivation of the retinal pigment hormone by chymotrypsin than by trypsin, but, because we do not yet know with any certainty the chemical nature of retinal pigment hormone, discussion of differences between trypsin and chymotrypsin in their proteolytic action on specific substrate linkages would be little more than speculation at this time. Such differences between trypsin and chymotrypsin may be due to the presence in crude eyestalk extracts of substances differentially inhibiting the two enzymes. Knowles *et al.* (1956) explain the failure of trypsin to inactivate chromatophorotropic hormone in eyestalk extract of *Palaemon* as probably due to inhibitory substances in extract of whole eyestalks, since electrophoretically separated chromatophorotropin is readily inactivated by trypsin. *In vitro* inactivation of chromatophorotropic hormone of *Uca* by trypsin, chymotrypsin, and papain has been reported (Pérez-Gonzáles, 1957; Stephens and Green, 1958).

On the basis of these and other properties, it has been suggested that the activity of chromatophorotropins is dependent on the presence of peptide bonds in the hormone, but the known esterase activity of trypsin and chymotrypsin do not permit this identification with assurance. Similar properties of the retinal pigment hormone, such as small molecular size, thermostability, inactivation by tissue extracts (peptidases?) and by proteolytic enzymes may indicate linkages common to the molecular structure of the two groups of hormones. The partial degradation by trypsin and the gradual "spontaneous" inactivation occurring in eyestalk extracts imply that portions of the hormone molecule may not be essential to physiological activity of retinal pigment hormone. This too must remain as speculation until such properties can be examined in highly purified preparations of the hormone.

SUMMARY

1. A standard assay for the content of light-adapting distal retinal pigment hormone in crustacean eyestalk extracts is described. Linear regression equations for the relation between response of the retinal effectors of test *Palaemonetes* and concentration of eyestalk extract from *Palaemonetes* and from *Libinia* have been calculated.

2. A *Palaemonetes unit* of this hormone is defined as that concentration of eyestalk extract, injected into a minimum of 10 dark-adapted *P. vulgaris* measuring 35–40 mm. in rostrum-telson length, which will result in an average distal retinal pigment index of 0.150. For the *Palaemonetes* and *Libinia* used in this study 1 *Palaemonetes unit* is equivalent respectively to concentrations of 3.5 and 0.34 eyestalks per 1.0 ml.

3. Thermostability, small molecular size, complete or partial inactivation by tissue extracts (peptidases?) and by crystalline trypsin and chymotrypsin are properties of the hormone consistent with a possible peptide structure.

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A SEROLOGICAL COMPARISON OF FIVE SPECIES OF ATLANTIC CLUPEOID FISHES

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The potential role of serology in fishery biology has received increased attention during the past decade (Cushing, 1952, 1956; Ridgway, 1957; Ridgway, Cushing and Durall, 1958; Sindermann, 1958; Suzuki, Shimizu and Morio, 1958; O'Rourke, 1959; Sindermann and Mairs, 1959; O'Rourke, 1960; Ridgway and Klontz, 1960). While most of this recent interest has been stimulated by application of red cell techniques to the identification of subpopulations, use of the varied methods available to serology can provide information on any taxonomic level.

Comparative serological studies of serum proteins began over half a century ago with the work of Nutall (1901) and have been pursued vigorously in recent decades by Boyden (1926, 1942, 1943, 1954), Gemeroy (1943), Leone (1949, 1950, 1954), Leone and Pryor (1954), Leone and Wiens (1956) and others. This work has demonstrated the utility of serum proteins in systematic studies, especially of higher taxonomic groups. Quantitative examination of the serological relationships of several species within a genus or family have been made (Stallcup, 1954; Leone and Wiens, 1955). Clear distinctions of species are possible with highly specific antisera; even species hybrids, such as the hinny and mule, may be distinguished from parental species (Boyden, 1942, 1953). Comparative serological studies using erythrocyte antigens have been most useful in distinguishing geographic groups, strains, subpopulations or races within a species (Owen, Stormont and Irwin, 1947; Moody, 1948; Ridgway and Klontz, 1960; and others). Irwin, Cole and Gordon (1936) and Irwin (1938, 1955) determined serological relationships of avian species and species hybrids using cellular antigens.

The present investigation was undertaken in 1958 to clarify the natural relationships of several clupeoid species from the western North Atlantic, as well as to establish a serological baseline for concurrent studies of intraspecies groups of herring. The following clupeoids were compared: alewife, *Alosa pseudoharengus* (Wilson); blueback herring, *Alosa aestivalis* (Mitchill); American shad, *Alosa sapidissima* (Wilson); Atlantic herring, *Clupea harengus harengus* Linnaeus; Atlantic menhaden, *Brevoortia tyrannus* (Latrobe). Four methods were used: (1) precipitin tests read photoelectrically with a Libby photronreflectometer; (2) precipitin tests visualized by agar diffusion; (3) erythrocyte agglutination with absorbed antisera; and (4) paper electrophoresis. This composite approach permitted scrutiny of species relationships from several serological viewpoints.

MATERIALS AND METHODS

Blood samples were collected from fish caught in commercial trap nets on the New Jersey coast. The sampling was done in the spring, summer and fall of 1958, spring and winter of 1959, and spring of 1960, using the cardiac puncture method described by Perkins (1957). Samples were held at approximately 4° C. until clotting had occurred; the serum was then decanted and either used immediately or stored at −20° C. Cells for antiserum absorptions and agglutination tests were washed from the clots remaining.

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1. *Photronreflectometer*

Since photronreflectometer determinations and agar diffusion are but different methods of visualizing precipitin reactions, the same rabbit antisera could be used for both. Pooled serum samples constituted the antigens for each species of fish tested. Antisera were prepared in rabbits by six or twelve subcutaneous injections of .5 ml. of antigen given on alternate days. Only a single series of antigen injections was given, to obtain as specific antisera as possible, in accord with findings of Wolfe and Dilks (1946) and Leone (1952). This resulted in antisera of somewhat lower titer, which are subject to relatively larger experimental error when tested than are strongly reacting antisera. However, an attempt was made to reduce any such error by repeating tests of each serum-antiserum combination. Photronreflectometer determinations were made with a 20-minute reaction time, which provides greatest separation of antigens from closely-related species (Leone 1950). Turbidimetric precipitin tests were carried out following the methods outlined by Boyden and DeFalco (1943) and Leone (1949). Constant amounts of antiserum were added to doubling dilutions of antigen, 1:250 to 1:625,000. This provided a titration curve with a peak at optimal proportions of the reactants, and with slopes to extreme antigen excess on one side and extreme antibody excess on the other. Whole curve comparisons of reactions were made, using summated turbidities of all the antigen concentrations tested, this summation being proportional to the area subtended by the curve. The homologous reaction was designated as 100%, so the summated turbidity (relative area) of the curve obtained with each heterologous antigen could thus be related to the homologous reaction as a per cent. Since five species were compared, five curves were obtained for each antiserum.

2. *Agar diffusion*

The agar diffusion technique was essentially the method originally described by Ouchterlony in 1948, and since used by Bjorklund (1952a, 1952b), Leone, Leonard and Pryor (1955), Casman (1958), Wilson (1958), Morrill (1959), Ridgway (personal correspondence) and others. A medium of 1.5% Difco agar and .72% sodium chloride was prepared and a basal layer of 8 ml. poured into a flat-bottomed Petri dish. After the basal layer hardened, cylinders of glass tubing (1 cm. in length, 4 mm. inside diameter) were placed in position, one in the center for antiserum and others set equidistantly around it, for the sera of the five species. The center of each serum receptacle was 1.5 cm. from the center of the antiserum receptacle. After a second 8-ml. layer of medium had been added and hardened, undiluted antisera and pooled sera were placed in the cylinders in amounts of .25 ml. per receptacle. The plates were then sealed with masking tape, labelled, and incubated at a temperature of 35.5° C. for seven days. Reading of the precipitate patterns was visual, although photographs were made of some plates.

3. *Erythrocyte agglutination*

Three rabbits were immunized with pooled washed cells of each species tested. Six or nine .5-ml. doses were injected subcutaneously on alternate days. Trial bleedings were made 10 days after the last injection and if the antiserum titer was adequate, food was withheld and the animals bled terminally on the following day. Antisera were frozen in 3-ml. aliquots at −20° C. until absorptions and cell agglutination tests were carried out.

Cells of each clupeoid species used for antiserum absorptions were washed three times in 1.5% saline, and approximately equal amounts of erythrocytes from all individuals in the sample were pooled. Antiserum was diluted 1:4 and added to pooled cells in proportions of four parts antiserum to one part cells. After 10 minutes of absorption the cell-antiserum suspension was centrifuged and the absorbed antiserum tested against a previously removed aliquot of cells used for absorption. Absorptions of antisera to all five species with each of the five erythrocyte pools were done simultaneously, and one absorption was usually sufficient to remove all antibodies reactive with the absorbing cells. Agglutination tests were then carried out, using all possible combinations of absorbed antisera and cells from each of the five species. Each agglutination test used .2 ml. absorbed antiserum and .05 ml. of 4% cell suspension. Readings were taken after 15 minutes of incubation at room temperature and 30 seconds' centrifugation.

4. *Electrophoresis*

Paper electrophoresis was carried out with a Spinco Model R system for six hours at 15 milliamperes, using a veronal buffer of pH 8.6 and ionic strength of 0.05. With each run, a sample of human serum was used as a control. Each sample consisted of .01 ml. of serum. Representative curves were obtained by use of a photoelectric densitometer.

RESULTS

1. *Photronreflectometer*

The results of serological comparisons of five species of clupeoids, using the photronreflectometer with one series of highly specific antisera, are summarized in Figure 1. Comparative values for reactions of each antiserum are in vertical columns beside each curve. Heterologous antigen reactions are presented in per cent of the homologous reaction. Reciprocal relationships show good agreement in most cases, especially in view of the fact that each antiserum must be considered as a separate entity as far as its specificity (discriminating capacity) is concerned.

The serological relationships of the five species presented in Figure 1 are obviously not linear, but may be expressed satisfactorily in a three-dimensional graph (Fig. 2) based on "serological distances" calculated by subtracting the per cent heterologous reaction from 100 (Boyden, 1926). This converts the relative correspondence of that particular antigen to a "relative distance" value. With this method antigens closely related to the homologous antigen will be relatively close to it, while those antigens of increasing dissimilarity will be increasingly far from the homologous. For example, in Figure 1 the serum proteins of the alewife gave a reaction that was 90% of the homologous reaction between blueback serum and

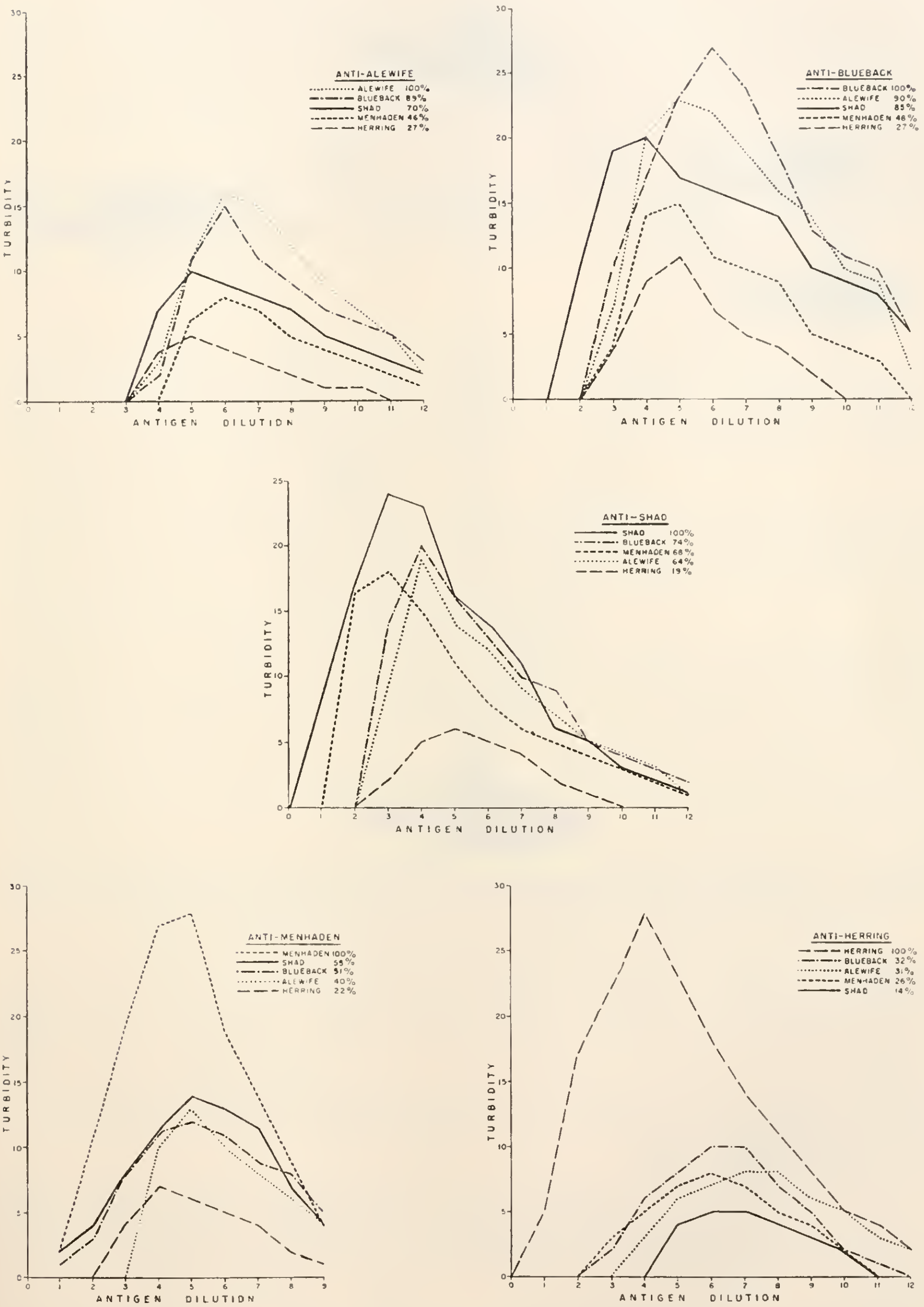


FIGURE 1. Precipitin curves derived from reactions of serum antigens of five clupeoid species with antisera prepared to each species.

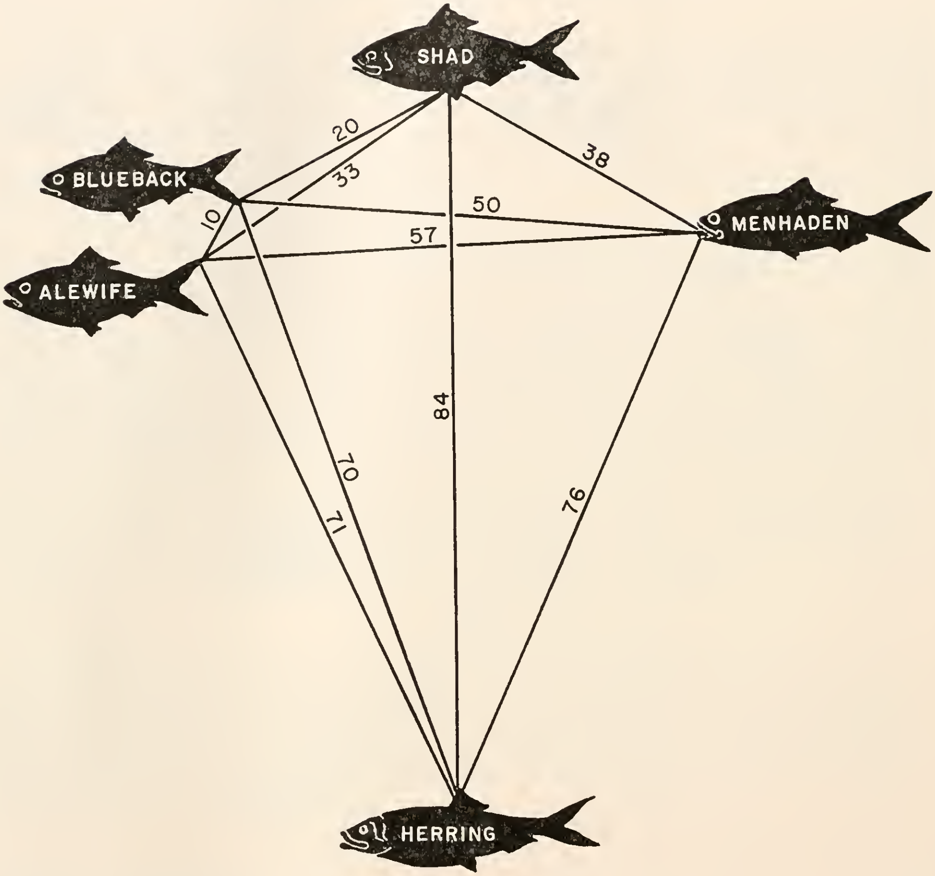


FIGURE 2. Three-dimensional representation of the present relationships of five clupeoid species, as determined from precipitin tests read with the photronreflectometer.

anti-blueback serum. The relative serological distance between blueback and alewife with this antiserum is thus 10. However, the reaction of herring serum proteins with the anti-blueback serum was only 27% of the homologous, so the relative serological distance between blueback and herring with this antiserum is 73. All relative distance values between any two species were averaged in preparation of Figure 2, and each species locus was determined by average distance values from each of the other species.

The data indicate that blueback and alewife have a high degree of serological correspondence; that shad are closer to the blueback-alewife complex than to either menhaden or herring; and that menhaden and herring are remote from all others, with herring consistently at the greatest serological distance from the other four species.

The numerical relationship values obtained are not fixed, but reflect results obtained with the particular antisera used. The relative positions of species with respect to one another should, however, remain relatively constant. Boyden, DeFalco and Gemeroy (1951) and Boyden (1953) have demonstrated that despite variations in specificity and systematic range of several antisera of the same kind, a consistent placement series will emerge for the species tested. This was demonstrated in the present work by a second series of antisera with lower specificity obtained by deliberately prolonged injections extending over a one-month period. Such antisera were in most instances less specific, but no variations in relative placement of the five species occurred. Serological distances separating heterologous and homologous species were reduced in most cases, but relative positions and placements were similar.

2. *Agar diffusion*

Clear patterns of precipitate were obtained with each of the antisera used. Although minor differences were noticed in the discriminating capacity of different rabbits immunized against the same species, the reproducibility was high with respect to number and position of precipitate bands in the gel. Diagrammatic sketches of typical patterns appear in Figure 3.

Since results of agar plate tests are very difficult to quantitate, they are not as useful for the determination of exact serological distances between species as the photronreflectometer. However, since closely related species display similar reactions and share some precipitate bands in reactions of identity, it is possible to get a good general idea of the relationships involved.

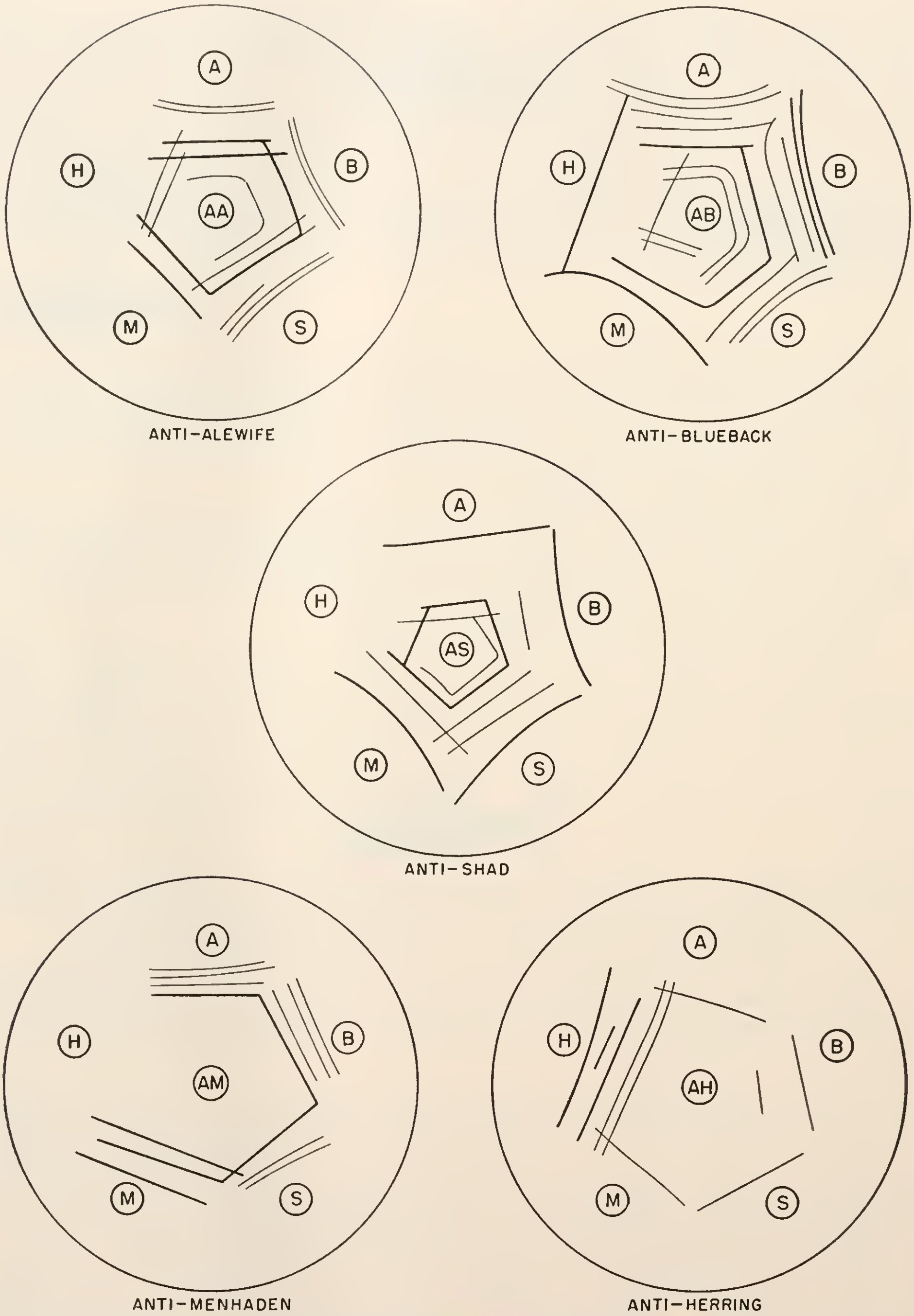
Alewife and blueback sera showed very similar reciprocal reactions. Shad serum reacted strongly to antisera prepared against alewives and bluebacks and shared some precipitate bands with these species, but the reciprocal reactions were somewhat weaker; thus, in the overall picture shad must be placed slightly farther from alewives and bluebacks than these two species are from one another. Menhaden and herring appear to stand somewhat apart from the alewife-blueback-shad complex, but the reactions of menhaden showed a closer relationship to that group than did those of herring. Apparently, menhaden and herring have very little taxonomic affinity, for antisera prepared against either of these species evoked only the faintest traces of precipitate from sera of the other, and these trace reactions were always of the type representing complete non-identity of antigens.

The results of the agar diffusion tests substantiate the more precise picture of relationships shown by the photronreflectometer. The positions of the species are in the same orientation as those deduced from the turbidimetric measurements.

3. *Erythrocyte agglutinations*

Tests were made with reagents obtained by absorbing each antiserum with cells of each of the five species. The reactions (Table I) represent composites of three separate tests, each using different samples of fish blood and a different antiserum. Results are recorded conventionally in descending order from (++++), representing complete agglutination, to (−) representing no agglutination.

The reduction in strength of agglutinations after absorptions indicates that all antisera contained substantial amounts of cross-reactive antibodies. Alewife and blueback cells shared many antigens, so that absorptions with either gave very similar but not identical reactions. Shad and menhaden shared some antigens, so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ANTI-ALEWIFE

ANTI-BLUEBACK

ANTI-SHAD

ANTI-MENHADEN

ANTI-HERRING

FIGURE 3. Diagrams of reactions in representative agar diffusion plates for each of the five clupeoid species. A = alewife, B = blueback, H = herring, M = menhaden, S = shad.

Table I

Reactions of erythrocytes of five clupeoid species with absorbed antisera

Antiserum	Cells used in absorptions	Agglutination reactions				
		Alewife	Blueback	Herring	Menhaden	Shad
Alewife	Alewife	–	–	–	–	–
	Blueback	+	–	+	–	–
	Herring	+++	++	–	–	+
	Menhaden	+++	++	++	–	–
	Shad	++	+	++	–	–
	Unabsorbed	+++	++	+++	+	++
Blueback	Alewife	–	–	+	–	–
	Blueback	–	–	–	–	–
	Herring	++	+	–	–	–
	Menhaden	++	+	+++	–	–
	Shad	++	+	++	–	–
	Unabsorbed	++++	+++	++++	+	++
Herring	Alewife	–	–	+	–	–
	Blueback	+	–	++	–	–
	Herring	–	–	–	–	–
	Menhaden	+	+	+++	–	–
	Shad	–	–	++	–	–
	Unabsorbed	++++	++	+++	+	++
Menhaden	Alewife	–	–	++	+	+
	Blueback	+	–	++	+	+
	Herring	+	+	–	+	+
	Menhaden	–	–	–	–	–
	Shad	+	+	++	+	–
	Unabsorbed	+++	++	+++	++	++
Shad	Alewife	–	–	+++	+	++
	Blueback	+	–	+++	++	++
	Herring	+	–	–	++	++
	Menhaden	+++	++	+++	–	+
	Shad	–	–	–	–	–
	Unabsorbed	+++	++	+++	+++	+++

that absorptions gave a somewhat similar pattern of reactions, although the relationship was by no means as close as that of alewife and blueback.

4. Electrophoresis

Electrophoretic analyses were made with both pooled and individual sera. Individual sera appeared in all cases to give better definition of components, although they conformed to the general pattern of the pools. Considerable intraspecific variation in patterns was evident, but a generalization of each species pattern was possible. The curves derived from these generalized patterns are depicted in Figure 4, as determined by densitometer readings; they are, however, not intended to illustrate absolute species specificity. The numbers designating each peak are

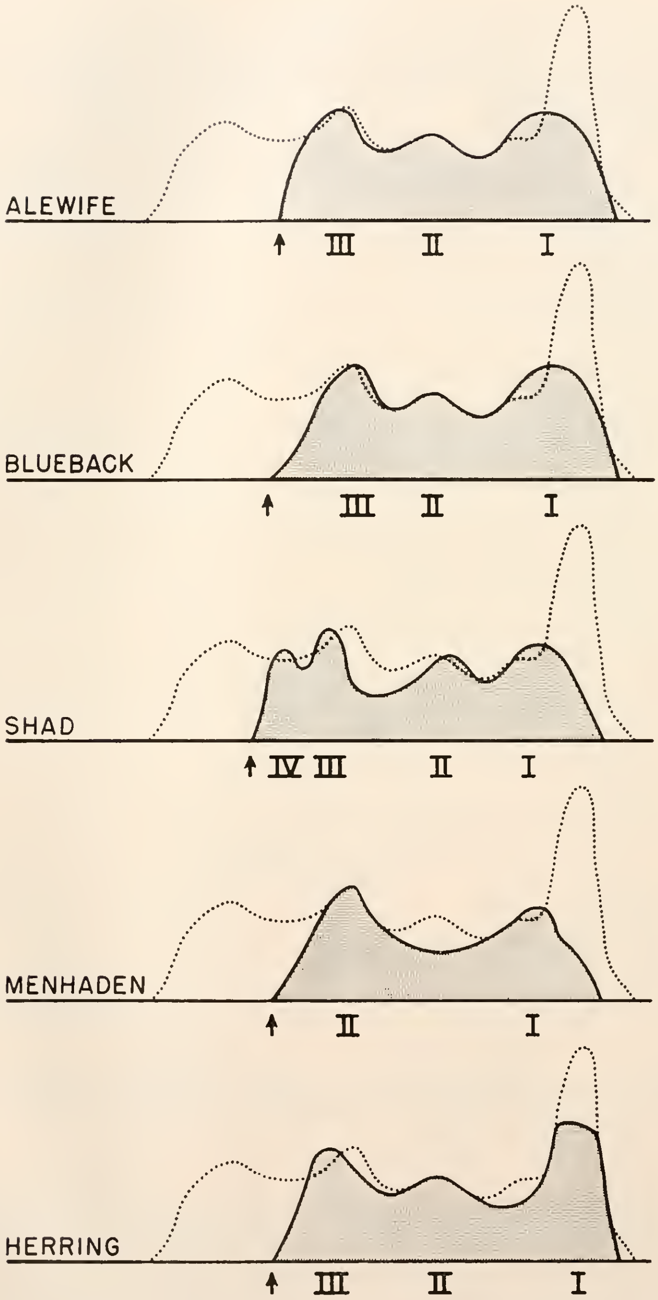

Figure 4. Representative paper electrophoresis diagrams for five clupeoid species as determined by densitometer readings. Dotted line represents human serum used as control.

used for ease in description and do not denote identity of components between species.

The alewife and blueback sera displayed very similar electrophoretic patterns, each with three major fractions. The main difference between the two species was found in the least mobile fraction; in alewives this fraction was less mobile than human beta globulin, whereas in bluebacks it migrated further than human beta globulin. Both species had a weak fraction of the same mobility as human alpha-2 globulin, and a fraction of slightly less mobility than human albumin. In some specimens of alewife, a weak fourth fraction appeared, intermediate in mobility between the human alpha-2 and beta globulin fractions.

The shad samples tested displayed a pattern of four fractions. The least mobile of these did not migrate as far from the point of application as human beta globulin. A strong fraction with slightly more mobility was present in all specimens, as were moderate fractions with slightly more mobility, respectively, than human alpha-2 and alpha-1 globulins.

The pattern shown by the menhaden sampled had two fairly strong fractions. One of these was identical in mobility to human beta globulin, and one was intermediate between human alpha-1 globulin and albumin.

Three moderately strong fractions were obvious in the patterns of the sea herring tested. The least mobile migrated a shorter distance than human beta globulin, while the others travelled the same distances, respectively, as human alpha-2 globulin and albumin. In some fish of this species a fourth fraction appeared which was intermediate between human alpha-1 globulin and albumin.

Discussion

1. *Photronreflectometer*

The clear differentiation of five clupeoid species with this precipitin technique read photoelectrically offers tempting possibilities for future studies. It would be interesting, with the background of the present data, to compare other morphologically similar but geographically isolated clupeoid species. Suspected hybrids, such as those between *Alosa pseudoharengus* and *A. aestivalis* might also be examined serologically. Comparison of populations or "races" of cosmopolitan species, such as *Clupea harengus,* with serum techniques might also prove instructive.

The limitations of such studies must be kept in mind. Results of serological examinations would not be considered as the sole criterion for taxonomic conclusions, but should be evaluated together with morphological and other data to provide as broad a base as possible for such conclusions. Information about the possible influences of environmental and physiological changes on serological reactions should be obtained. It should also be emphasized that results will indicate present serological relationships, but will give only indirect information about the evolutionary history of the species concerned. Despite such limitations, serum techniques offer possibilities in fisheries research that should be explored as vigorously as other techniques for the understanding of natural relationships.

2. *Agar diffusion*

Further experimentation should be conducted with agar diffusion to determine its usefulness in studies on fish populations. Absorption of antisera to remove species antibodies would probably be a virtual necessity for work at the intraspecific level.

The advantage of this type of test obviously lies in the fact that the total precipitate is resolved into its component antigen-antibody reactions, which are subject to direct visual observation. While this permits a high degree of qualitative differentiation and gives a good idea of general species relationship, it is very difficult to measure exactly the total amount of precipitate formed. For this reason, it is best to leave the final calculation of relative serological distances between species to turbidimetric methods, keeping in mind the fact that rabbits or other experimental animals are not the exclusive and final arbiters of natural relationships.

3. *Erythrocyte agglutination*

The use of pooled cells to absorb antisera to all five clupeoids, and the subsequent testing of such pools with reagents obtained by absorptions, have provided a criterion for determining the relationships of five clupeoid species. It would be instructive to apply this test to additional clupeoid species in other regions.

Another logical extension of this work would be determination of the distribution of similar or identical antigens in individual blood samples of the five species. Individual differences have been noted in the present work for all five species, but, except for herring, blood group systems have not been proposed. The use of pooled cells masks individual differences, and it is obviously less precise than a study of discrete antigens with specific reagents. To test the utility of the single antigen approach, individual samples of all species were tested with the reagents used in routine examination for the C blood group antigen of herring (Sindermann and Mairs, 1959). Positive reactions were obtained with most alewives, half of the bluebacks, a few shad, and no menhaden, indicating the existence of the same or at least a closely related antigen in species other than herring. Similar tests with other specific reagents could create a mosaic of reactions that would provide a clearer picture of the affinities disclosed by the present study. The utility of such an approach has already been demonstrated with certain mammalian species by Stormont and Suzuki (1958).

4. *Electrophoresis*

Although it was possible to construct electrophoretic patterns characteristic of the five species of clupeoids, the intraspecific variations encountered in this study and in an electrophoretic examination of herring populations (Mairs and Sindermann, 1960) indicate that great caution should be employed in the establishment of specific patterns for teleostean species. It has been shown that the component fractions of serum display variability in both quantity and electrophoretic mobility depending on such factors as disease, age, sex and starvation (Moore, 1945; Dessauer and Fox, 1956; Drilhon *et al.*, 1956; Sindermann and Mairs, 1958). This variability could lead to significant overlap and confusion of patterns, especially among closely related species. In many cases, it is doubtless possible to characterize a high proportion of the electrophoretic patterns in a sample, but proposal of a species-specific pattern would have to follow testing of a large number of individuals, with close reference to maturity, disease, and any other physiological or environmental factor known or likely to affect electrophoretic characteristics. Only in this way could a species "norm," if such exists, be properly defined and an insight gained on the true significance of the variations encountered.

The present study indicates that, while paper electrophoresis may have some general usefulness in fish taxonomy, immunological techniques are preferable for precise differentiation and attempts at determining natural relationships.

Summary and Conclusions

1. An investigation of the serological relationships of five species of clupeoid fishes was made by four methods: (a) photronreflectometer, (b) agar diffusion, (c) erythrocyte agglutination with absorbed antisera, and (d) paper electrophoresis.

2. Agar diffusion enabled qualitative differentiation of the species tested, while the photronreflectometer provided a quantitative measure of relative serological distances between species; results from the two methods were in good agreement.

3. On the basis of results obtained by the photronreflectometer and agar diffusion methods, the following species relationships are indicated: alewives and bluebacks are very closely related; shad lie quite close to alewives and bluebacks, but farther from them than alewives and bluebacks are from one another; menhaden and herring are further removed from the alewife-blueback-shad complex, with menhaden closer to it than herring; herring are comparatively remote from the other four species.

4. Generalized electrophoretic patterns were found for each clupeoid species. However, because of intraspecies variability, paper electrophoresis does not seem to be as useful a procedure for determining relationships of fishes as immunological methods. Species-specific patterns should be proposed only after large numbers of individuals representing both sexes have been sampled under different physiological conditions.

5. Absorptions of rabbit antisera with pooled erythrocytes of each of the five clupeoid species indicated that alewife and blueback were antigenically very similar; menhaden and shad were antigenically somewhat similar, although not as close as alewife and blueback.

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REPRODUCTIVE BIOLOGY OF LYCHAS TRICARINATUS (SIMON)[1]

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In a previous paper on the embryonic nutrition in *Lychas tricarinatus* (Mathew, 1960), I had drawn attention to a curious phenomenon of arrest of development in internal embryos in this scorpion. As this phenomenon was an unusual one, further studies were made on it and this paper embodies the results of these investigations.

MATERIALS AND METHODS

Gravid females of *L. tricarinatus* were collected almost every month from Mukkunni hills near Trivandrum (only during the wet season) and from Nagercoil and adjoining hills like Asambu and Thadikarankonam in Kanyakumari District of Madras State. Specimens were also collected from Vaikom and Moovatupuzha in North Travancore. Suitable cages were improvised for them and they were reared in these and observations were made in each case as to the delivery of young. As occasions demanded, the specimens were killed and the conditions of the ovary examined, and all these ovaries were preserved and labelled for future study.

OBSERVATIONS

The ovary consists of a pair of longitudinal tubes on each side, each pair connected together by five transverse tubes forming four quadrilateral meshes on each side; so, unlike other scorpions, here the female system has almost the same arrangement of tubules as in the male system. However, at the hindermost part, the inner longitudinal tubes of either side are fused so that this hind extremity of the ovary alone resembles the ovarian network of other scorpions. The developing embryos are inside the ovarian tubes as typical of the Buthidae. All along the outside of the tubes there are eggs in different stages of growth, in the tiny bubble-like follicles. Among these are a few which are larger than the others. The eggs in them are surrounded by thick envelopes. They have already begun to develop and in some, well developed blastoderms may be seen. Does this mean that while one set of embryos has already begun to develop inside the ovarian tubes, other sets, too, are developing, so that at one time there can be young of different stages of development in the ovarian tube? On morphological and physiological grounds, this is an impossibility and does not happen in any scorpion studied so far. Since ovarian tubes themselves are the embryo-ducts, if there are embryos of different stages of development in these tubes, when some of the embryos mature and are extruded, they will push out the younger immature embryos too.

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Ovarian tubes in which the embryos are fully mature also have eggs in different stages of growth, in tiny bubble-like follicles, with a few larger follicles enclosing segmenting zygotes or fully formed blastoderms, on the outside; just the same as seen in the earlier stages. Evidently they have remained all through the development of the internal embryos without any change. This would indicate that these zygotes and early embryos outside the ovarian tube are held in arrested state of growth, or in a condition resembling diapause, until the extrusion of the developed embryos. After the extrusion of these developed embryos, the suppressed embryos might get pushed into the ovarian tube and resume their course of development. If this should happen, the scorpion, after giving birth to one brood, must be able to develop another brood of embryos without fresh copulation. To test if this actually happens a few breeding experiments were conducted and observations were made.

Breeding Experiment

Six gravid females were selected from a collection from the field and reared in artificial cages providing as nearly natural conditions as possible—one female in a cage. They were properly fed, given insects and water, and observations were recorded.

Female A

Collected and caged	3–5–1958
Delivered the young	9–5–1958
Young moulted	14–5–1958
Young left the mother	19–5–1958

After delivery the mother was kept in the cage and properly looked after. It grew well and after about a month was seen with the abdomen swollen as if it was becoming gravid again. There was no male scorpion anywhere in the vicinity. A little later the abdomen was still more distended and through the thin translucent sterna, the internal embryos could be seen quite clearly. There was no more doubt as to its second pregnancy. On June 20th, *i.e.*, 41 days after the previous extrusion of young, a second brood came out and the young took their position on the mother's back as usual.

Delivery of the second brood	20–6–1958
Young moulted	26–6–1958
Young left the mother	1–7–1958

The female was then transferred to another cage and carefully looked after for another month. By this time it was showing considerable weakness and it was not possible to keep it alive any further. So it was killed on the 28th of July, 1958, and the ovary was examined. There were 27 developing embryos inside, which were fairly advanced and the black pigmented eyes were conspicuous.

With three of the other specimens this experiment was repeated under similar conditions and the above observations were confirmed. Since then these observations were further repeated and confirmed in 1960 and again in 1961 with fresh specimens obtained mostly from Nagelcoil. From these rearing experiments, the following conclusions can be drawn: (1) *Lychas tricarinatus* can, after one delivery, produce at least three successive broods of young when isolated from all possibility of contact with a male. (2) The time elapsing between one delivery and the next is about 40–42 days. This may be taken as the approximate period of gestation. Since the period of gestation can be approximately determined, counting from the time of one delivery, it is possible to keep females in captivity and study the stages of development of embryos within specified periods of time. So a few gravid females were kept in captivity and after one delivery, the embryos were examined and studied at different intervals. In all these there were growing embryos developing at the normal rates, as could be tested by the condition of the embryos at definite periods in the different specimens examined. The development and the stages attained as seen in these studies will be described elsewhere, dealing with the embryology of *Lychas*.

The series of breeding experiments described above show conclusively that this scorpion does produce successive broods without pairing after each brood has been extruded. How are the new embryos formed? The following possibilities may be suggested, but each has to be tested and proved.

(1) It may be that there are slight variations in the developmental stages attained by embryos of a brood in the uterine tube and the most mature are extruded first and the others are retained till they too are fully developed, to be extruded as the second brood, and so on.

(2) Parthenogenesis.

(3) Sperm received at one copulation are preserved in the spermathecae, viable and capable of fertilising eggs for future broods.

(4) After once pairing, numerous eggs are fertilised but only a few of these can develop in the limited space of the uterine tubes (ovarian tubes). These few alone pass into the uterine tubes and continue development while the others are kept in an arrested state of development till the developing brood is extruded.

Regarding the first possibility, suggested above, we may note the following facts and observations: (1) Opening gravid females, we do not notice such disparity in stages of the development of individuals of a brood. In early stages a slight disparity may appear but by the time the embryos have attained about half maturity, all are of the same stage except rarely a diseased or injured individual which fails to be extruded. (2) When a female is opened soon after extrusion of a brood, no younger stages are seen to be retained in the ovarian tubes but they are completely empty and lie flabby in the body cavity; all eggs and zygotes on the ovarian tubes are on the outside only.

Occasionally, when several specimens are opened, one comes across a dead embryo still retained in some part of the ovarian tube. But this is a diseased or dead one which could not be extruded and so has been retained there to undergo gradual histolysis and absorption. These have been observed in other scorpions too, such as *Heterometrus* (Mathew, 1956). This may cause obstruction for the other embryos, but because of the retiform arrangement of the ovarian tubes providing several alternating pathways, no such blockade actually takes place; all viable embryos escape.

These considerations help us to rule out the first possibility.

The second possibility suggested is parthenogenesis. It is known to take place under special conditions in many arthropods, but one indication for parthenogenesis is the absence or rarity of males. With regard to *Lychas,* however, this condition does not hold. Estimating the proportion of the sexes from collections that have been made during these four years, one can say that in number the sexes are almost equal—sometimes there may be a slight predominance of females. At any rate it can be most confidently stated that there is no scarcity of males.

Again, if development is parthenogenetic there is no need for the early formation of zygotes and blastoderms; the development processes may well start after one brood has been extruded.

The third possibility suggested is that viable sperm may be retained in the spermatheca to be used over and over again. This is not an impossibility but microscopic examination of the contents of the seminal vesicles has failed to show sperms held in reserve. Also, as already observed with reference to parthenogenesis, if there is sperm held in reserve for future fertilisation, why the early formation of zygotes and blastoderms?

The last suggestion, which is in agreement with all the observed phenomena, appears to be most satisfactory.

(1) The ovarian tubules of a female which has recently extruded a brood of young possess numerous tiny bubble-like follicles in which there are young ova or zygotes or blastoderms, the latter two being distinguishable by their larger size and being covered over by a thick pigmented shell, the "chorion."

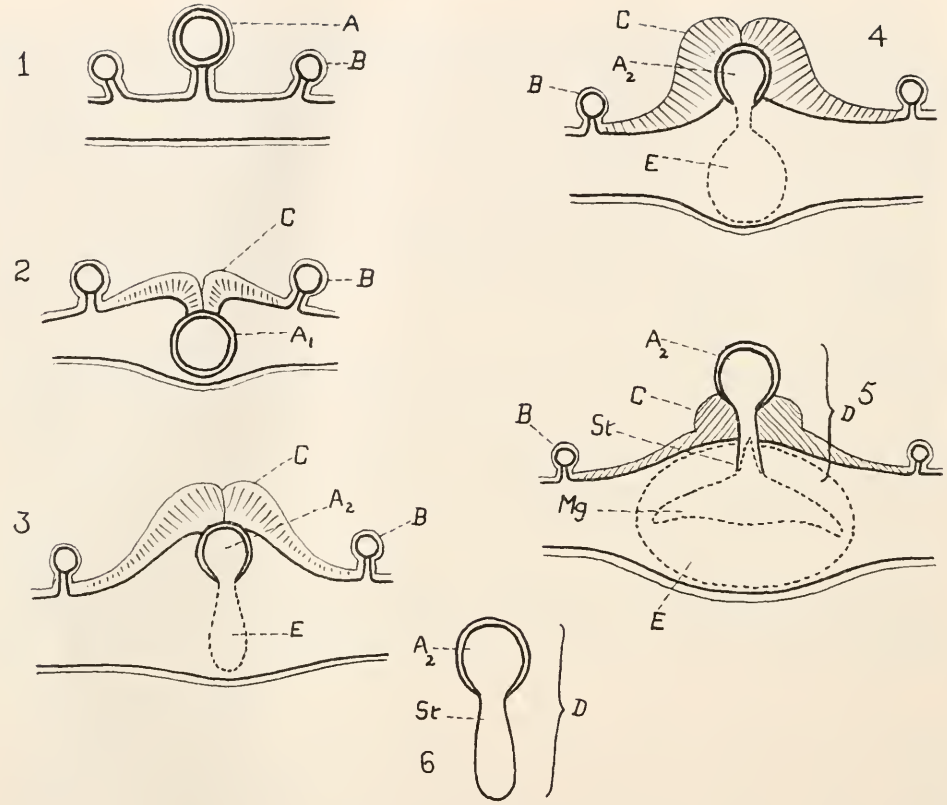
(2) In the ovarian tubes, examined a week later, we find that some of these blastoderms enclosed in the "chorion" have sunk into the ovarian tube while the zygotes that are still outside begin segmentation and develop into blastoderms. Only a limited number of embryos can sink into the ovarian tube and the number appears to be roughly controlled by the extent of space available on the ovarian tube. In an ovarian tube with well developed internal embryos, the swellings are seen more or less evenly spaced, thus giving it the characteristic moniliform appearance.

(3) All through the subsequent development, when the internal embryos are growing to maturity, the external zygotes and blastoderms remain without any further development. They remain dormant till this brood is extruded.

Embryonic Diapause in Lychas tricarinatus

This arrested development described above, as seen in *Lychas tricarinatus,* may be taken to be a special type of diapause of an internally developing embryo. It would be interesting to enquire into the probable mechanism of the initiation and final release of this "diapause." The special development of certain glandular structures synchronising with the initiation and release of diapause makes it highly probable that these are under hormonal control. The following observations of the developmental cycle, accompanied by the enlargment or breakdown of the glandular structures concerned, are illuminating.

Stage 1. In *Lychas,* the development of the embryo up to the establishment of the germinal layers take place in the follicles outside the ovarian tube. The zygotes and blastoderms are surrounded by a thick dark pigmented shell or "chorion" (Fig. 1, A).



KEY TO LETTERING

A, large follicle with blastoderm surrounded by chorion.
A1, egg with blastoderm sinking into the ovarian tube.
A2, "empty chorion" from which the embryonic rudiment has passed out. Develops into corpus luteum 2, forming the embryonic feeding mechanism.
B, small follicles with eggs (earlier).
C, cells at the base of the follicle become glandular corpus luteum 1.
D, the cast off chorion and stalk—corpus luteum 3.
E, embryo.
Mg, midgut of the embryo.
St, stalk of the feeding mechanism connected with the embryonic gut.

FIGURE 1. Part of the ovarian tube (early) bearing follicles enclosing blastoderms or eggs.

FIGURE 2. A follicle with blastoderm sinking into the ovarian tube (A1), causing the part of the ovarian tube to bulge a little. At the site of the insinking (C, C) the outer cells begin to enlarge and become glandular.

FIGURE 3. The embryonic rudiment (E) is pushed into the ovarian tube from the ruptured "chorion" (A2), which is at the same time pushed upwards, everting the invaginated outer layer at the site of the insinking of the egg. The cells of this portion (C) have now become enlarged and glandular. At this stage they form button-like thickenings on the swollen parts of the ovarian tube in which are the developing embryos.

FIGURE 4. The upward movement of the chorion (A2) continues. It pushes itself out of the inner layer and projects out, covered over by the everted glandular cells of the follicle (C).

Stage 2. After extrusion of a brood, these follicular embryos surrounded by the "chorion," which appears to be developed out of follicular cells, sink into the ovarian tube (Fig. 2, A1). The site of the insinking marking the base of the follicle soon develops a spongy texture and becomes glandular (C in the figures). Soon this assumes the form of a button-like papilla with enlarged glandular cells. This structure may be regarded as *corpus luteum* 1. The term "corpus luteum" has been used by Laurie (1890) for the empty follicle in *Euscorpius* after the embryo has passed into the ovarian tube for further development. He surmised that the hormone from this corpus luteum prevents the development of new embryos. Here, however, it is not one structure that develops secretory properties in connection with the developmental cycle, but at least three different parts of follicular origin serve this function; these structures are here tentatively called corpus luteum 1, 2 and 3.

Stage 3. In subsequent development when the "embryonic rudiment" is passed out of the "chorion" into the ovarian tube, the partially empty "chorion" is pushed upwards through the spongy structure, that has been termed above corpus luteum 1, and breaking through it, gets exposed in the body cavity as a tiny rounded dark brown body, A2, retaining connection with the gut of the developing embryo (Fig. 5). This body with its stalk (St.) develops into the queer "feeding mechanism" which has been described elsewhere (Mathew, 1960). This structure, which also is of follicular origin, has been regarded as a corpus luteum and may be distinguished as corpus luteum 2 (A2). When this structure is fully formed, corpus luteum 1 is seen to form a spongy globular mass surrounding its stalk (Fig. 5. C.). As the embryo grows, it is seen to be fed with the rich secretion of the feeding mechanism, directly passed into the midgut of the embryo.

Stage 4. When the growth of the embryo is completed and it is about to be extruded, the connection with the midgut is constricted off where it joins the midgut and the whole stalk, tipped with the dark globular "chorion," slips off and drops into the mother's body cavity during parturition (Fig. 6).

Stage 5. Post-partum changes. The discarded feeding mechanism with its stalk (D), instead of immediately degenerating, develops into a club-shaped glandular organ, apparently actively functioning for some time. This may be called corpus luteum 3. Soon after, however, this structure begins to atrophy, undergoing histolysis.

DISCUSSION

From the above observations, it will be seen that corpus luteum 1 is formed immediately after the developing embryos have sunk into the ovarian tube. No more of the embryos sink in after this and so it is suggested that its secretion has the property of inhibiting further development of the follicular embryos: they remain outside the ovarian tube without sinking in and their growth appears to be arrested. In other words, they are in a state of "diapause" in which they remain till the developing embryos are extruded.

FIGURE 5. Finally the chorion (A2) breaks through the ovarian wall completely and projects out freely; the glandular cells (C) form a collar around the stalk (St) connecting the chorion with the gut of the embryo.

FIGURE 6. When parturition takes place, the chorion (A2) with its stalk (St) is separated off and left in the body cavity. There they develop into club-shaped bodies (D).

After the extrusion of the young, the feeding mechanism, consisting of the globular "chorion" and its club-shaped stalk, are released from the ovarian wall and drop into the body cavity. These curious bodies rapidly develop into glandular structures of a third type, called above corpus luteum 3. These are really spent structures which have served their purpose and are to disintegrate normally. But instead of that, they develop into glandular structures functioning actively for a time. To the secretion of this we can attribute only one function—that of "reviving" the embryos from their "diapause" condition, for, soon after they are released into the body cavity, fresh external embryos begin to sink in and continue development. At the same time it is significant that the glandular cells of corpus luteum 1, whose secretion was suggested to prevent further insinking of embryos, have shrunk up and become almost indistinct.

Soon after the "diapausing" or "resting" embryos have started their further course of development, we find that these bodies, corpus luteum 3, break up and disappear.

Here we have a singular instance of embryonic diapause in an internally developing egg. In insects, embryonic diapause is fairly well known; it occurs more commonly at stages more advanced than the blastoderm stage. But all these eggs are outside and their "diapause" and later its termination appear to be controlled by the influences of the external environment (Lees, 1955). In this scorpion, however, diapause occurs at the blastoderm stage or earlier (?), but never later. Further development of the blastoderm can take place only after it sinks into the ovarian tube, and later development appears to be an uninterrupted process. Naturally, the blastoderm stage is the most suitable period for an arrest of growth.

It is possible to look upon diapause in *Lychas*, also, as controlled by the environment—the maternal body cavity in which development takes place. Naturally it has not to depend on a precarious external environment but is under the influence of a well regulated internal environment changing rhythmically in correspondence with a reproductive cycle.

SUMMARY

1. An unusual instance of embryonic diapause in a scorpion is described. Among the eggs that are fertilised at a time, only a few sink into the ovarian tube and continue to develop to maturity while the others remain in a state of arrested growth or diapause, until the extrusion of the first batch of embryos. Further insinking of the eggs appears to be prevented by the secretion of corpus luteum 1.

2. After the extrusion of the first batch of embryos the diapausing embryos get revived and sink into the ovarian tube to give rise to the next brood. The termination of the "diapause," also, appears to be in response to a secretion from another glandular structure, termed here corpus luteum 3. This incidentally explains how *Lychas* can produce two or three broods in succession without undergoing repeated copulation.

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THE REPRODUCTIVE CYCLES OF THREE VIVIPAROUS TELEOSTS, ALLOOPHORUS ROBUSTUS, GOODEA LUITPOLDII AND NEOOPHORUS DIAZI[1]

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Extensive taxonomic and descriptive work has been done on the Goodeidae, a family of fresh-water viviparous cyprinodonts from Mexico, but very little is known about their reproductive cycles. Except for a detailed three-year laboratory study on *Neotoca bilineata* (Mendoza, 1939), no other major study on the reproductive cycles of the goodeids has been made either in the laboratory or in the field. The only other principal source of information on the breeding cycles in the family is in Meek (1904), in which a brief and inadequate statement is included in the taxonomic description of each species. Miscellaneous information on the reproductive cycles of the goodeids also is scattered in many of Turner's articles but he made no detailed study of any one species.

Because of the scarcity of this information, it was proposed to make an analysis, from field specimens, of the female reproductive cycles of three species in the family. This study concerns the duration of the female cycle, the number and size of broods, the length of the gestation period, etc. This is the first intensive study that has been made of the reproductive cycles from specimens collected in their natural habitat.

The writer is greatly indebted to Sr. Aurelio Solórzano Preciado, Director of the Estación Limnológica in Pátzcuaro, Michoacán, and to Juan Pizá M., one of the attendants at the station, for their unending cooperation in helping the writer to make many of these collections.

Materials and Methods

This study concerns three members of the family Goodeidae: *Alloophorus robustus* (Bean), *Goodea luitpoldii* (Therese von Bayern and Steindachner), and *Neoophorus diazi* (Meek). The three species were collected from Lake Pátzcuaro. They were chosen because they inhabit the same lake and because it was possible to get a continuous supply of adults throughout the year.

Mature *Alloophorus* and *Goodea* females normally range from 90–110 mm. in length, although exceptionally large specimens of each species may exceed 130 mm. Mature females of *Neoophorus* range from 70–90 mm. in length.

The writer relied entirely on local fishermen for aid in collecting specimens. Because of the exuberant growth of reeds, lilies and other vegetation near the shore, the local fishermen use the much-publicized "butterfly" nets as scoops and work from their small canoes in groups of three to five. In the open lake the fishermen use large seines that measure 300 feet or more in length.

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This study is based on a total of 3261 females collected during 1957; approximately 50 females of each species were collected twice each month throughout the year. Specimens were divided as follows: 1010 *Alloophorus,* 1117 *Goodea* and 1134 *Neoophorus.* Similar collections, numbering more than 6000 females, were made during 1956 and 1958 but these were used for reference and comparison purposes only. The average size of all collections was 47 ovaries. Some females were collected alive in the lake and others were purchased in the local market since fresh fish were available at least once per week. The fishermen normally bring in only mature adults; they show no preference for either sex or stage of gestation. *Alloophorus* and *Neoophorus* normally are collected along the shore of the lake; *Goodea* usually is collected in open water but may prefer the shore during the breeding season. Lastly, in obtaining specimens in the market, a definite effort was made not to select only large gravid specimens but to take all females, regardless of size or stage of gestation.

In these viviparous species, the ovary is a single structure, compact, spindle-shaped, hollow, and continuous caudad with the oviduct which in turn opens to the outside at the genital pore. The ovary has ovigerous tissue but it also acts as a uterus, for all development from fertilization to birth occurs in the ovarian lumen. When the young are ready for birth, they escape from the sacculated ovary and emerge as free-swimming forms.

Upon collection, each ovary was removed and preserved either in formalin or special fixatives such as Bouin's or Zenker's fluids. All ovaries collected were preserved, regardless of the stage of gestation, and placed in one of the following categories: immature ovaries, resting ovaries, ovaries with growing eggs, ovaries with free eggs, ovaries with embryos in different stages of development, and post-partum ovaries. On classifying the gonads into different stages, relatively little difficulty was encountered. Ovaries without free eggs or young were dehydrated and cleared in cedarwood oil. The gonads then were examined with a stereoscopic microscope and classified into the proper stage. Ovaries with embryos in actual stages of development were placed into one of twelve classes according to size. The smallest group ranged from 3.5–5.0 mm. in length and successive groups or classes were formed at increments of 2.5 mm. (*e.g.* 5.1–7.5 mm., 7.6–10.0 mm., etc.); the largest group was 30.1–32.5 mm. (in *Goodea*).

STAGES OF GESTATION

The data on the three species are given concurrently, that is, comparable stages of development are considered at one time for all three forms. The principal description is based on *Alloophorus* and is followed in turn by the ones on *Goodea* and *Neoophorus.*

Immature ovaries

Immature ovaries in all three species measured from 1–2 mm. in maximum width by 15–20 mm. in length. The typically immature ovary has delicate external walls and internal folds; eggs vary up to 350 μ and are densely packed in the anterior half or two-thirds of the gonad. Measurement of the diameter of the small ovaries was made with a micrometer eyepiece in a stereoscopic microscope; the length and diameter of the larger ovaries were measured with a millimeter ruler.

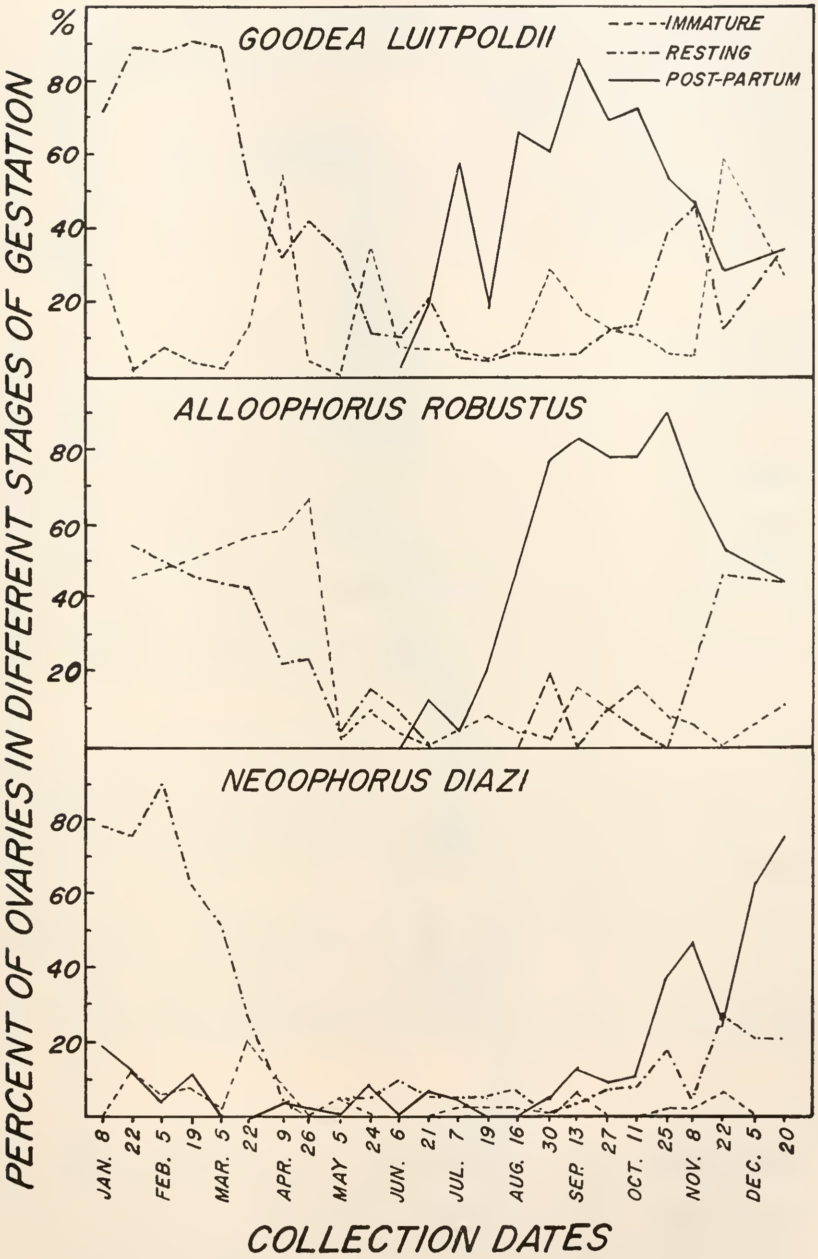

FIGURE 1. The occurrence during the year of females of the three species in immature, resting and post-partum stages. The number of ovaries in different stages appears as percentage figures of the day's collection.

Normally, few immature females were collected. Figure 1 shows that from January to April the number of these ovaries in *Alloophorus* is high, between 54.8 and 67.3% of the individual collections; thereafter the number drops markedly and remains low during the rest of the year. After spring very few immature females were captured. Figure 1 indicates that in *Goodea* immature ovaries did not follow quite the same pattern of appearance as those of *Alloophorus*. In *Neoophorus,* on the other hand, immature females appeared uniformly throughout the year, usually forming 10% or less of the collections. At no time were immature ovaries in this species as abundant nor did they have the pre-season high so clearly evident in *Alloophorus*.

Resting ovaries

Resting ovaries are mature but do not contain young. These ovaries vary from 2–3 mm. in diameter and up to 20 mm. in length in *Neoophorus* and 20–30 mm. in length in *Goodea* and *Alloophorus*. The external wall and internal folds are very thick. Eggs are few in number and vary in size but do not exceed 350 μ.

In all three species, the number of females in a resting condition is high during early spring but drops abruptly during early May in *Alloophorus* and *Goodea* and during early April in *Neoophorus* (see Figure 1). In general the number of resting ovaries decreases as the breeding activities start; thereafter the resting ovaries constitute a small but variable percentage of the collections until late summer, at which time breeding ceases. During winter the number of resting ovaries again rises and reaches a peak in the early spring, at which time breeding is resumed.

Ovaries with growing eggs

Ovaries in this condition resembled resting ovaries but differed in that eggs exceeded 350 μ and had grown to a maximum of 1 mm. in *Goodea* and *Alloophorus* and approximately .5 mm. in *Neoophorus*. All eggs in this category were still enclosed in a follicle embedded in the ovarian tissues. Measurement of the eggs was made with a micrometer eyepiece.

Growing eggs generally appeared during January and February although the precise time of appearance varied between the three species. If the sampling was representative, *Goodea* had a slightly longer period of egg growth than *Alloophorus*. Growth of new eggs stopped during June in both species and was not seen in later collections. Growth of eggs in *Neoophorus,* however, started at the same time but continued until the end of October.

Ovaries with free eggs

All ovaries with free eggs in the ovarian lumen, regardless of stage of development, were arbitrarily placed in this category. The eggs varied from stages near time of fertilization to stages with young approximately 3 mm. long. Such young were still enclosed within the egg membranes and were coiled around the yolk-like mass. At about this time (3 mm.) the young escape from the membranes and straighten out.

In *Goodea* and *Alloophorus,* ovaries with free eggs appeared in collections during April, May and June, a period of 2 to 2.5 months; thereafter, free eggs never appeared in the collections. In *Neoophorus,* ovaries with free eggs first appeared

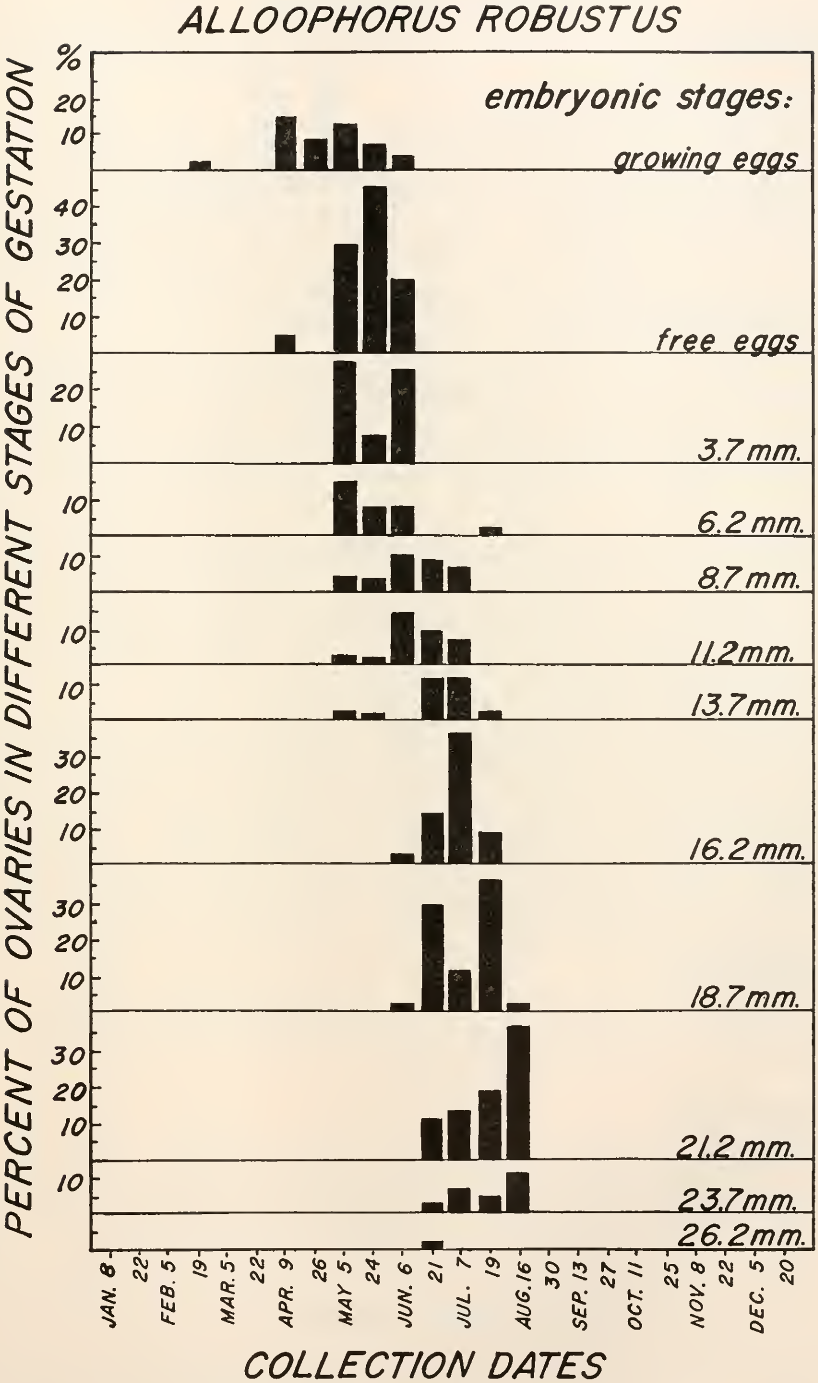

FIGURE 2. The appearance throughout the year of ovaries of *Alloophorus robustus* with embryos in different stages of development. For any one day, the bars represent the percentage of ovaries in each stage of gestation.

during February and thereafter were present continuously until November, at which time they disappeared.

Ovaries with young 3.5 to 32.5 mm. long

In all ovaries with measureable young the following procedures were employed. The diameter and length of each ovary were determined with a millimeter ruler. Following the measurement of the ovary, all young in each gonad were removed and thoroughly mixed in a petri dish. Four specimens then were chosen at random and measured with a ruler from the tip of the snout to the posterior edge of the caudal fin. The average of the four figures was derived and recorded as the size of the young in that particular ovary.

Development of the young was similar in both *Alloophorus* and *Goodea*. Embryos in the 3.5–5-mm. class appeared either in late April or early May. From this time on, developing young were found throughout the breeding season; reproduction ceased after August. While cessation was abrupt in *Alloophorus,* there were scattered females of *Goodea* that were late in their cycle as compared to the bulk of the population. For example, while most young of *Goodea* had reached a size of 23.7 mm. by the middle of July, one female was found on December 20 with young 23.7 mm. long. Figure 3 shows this and other similar examples. Most young of *Alloophorus* were born after they reached 21.2 mm. although some reached 26.2 mm. before birth. In *Goodea,* however, many young exceeded 21.2 mm.; some even reached a maximum of 31.2 mm. before birth. There is a noticeable temporal delay in the appearance of the larger sizes, that is, the larger the class size, the later the appearance of the embryos in the collections.

In *Neoophorus,* the reproductive period extended over eight or nine months. Embryos first appeared during the latter part of March and thereafter appeared continuously until January or February of the following year, at which time reproduction was suspended for a brief period of one or two months. Figure 4 shows embryos of maximum size were still present on December 20, 1957, the last collection of the year. Collections made during 1958 indicate that the 1957 breeding cycle did not terminate until February of 1958. It is similarly noted that one gravid female appeared in January 1, 1957; this female no doubt represents the end of the 1956 breeding season.

The collections of the three species made during the years of 1956 and 1958 strongly support the characteristics of the reproductive cycles as expressed during 1957.

Post-partum ovaries

This category identifies all ovaries from which young have recently been expelled. Immediately after the birth of young these ovaries appeared thin-walled and flaccid, the internal folds were thick and swollen and there were few eggs visible. Later, the ovarian tissues underwent regression but the ovary generally remained thick. Still later, the gonads again resumed the characteristics of a resting condition.

In *Alloophorus* the post-partum ovaries appeared on June 21 and thereafter appeared in large numbers in all collections until the end of the year (see Figure 1). After October 25 the number of these ovaries dropped, concomitant with the rise in the number of resting ovaries. At this transitional point it became more difficult

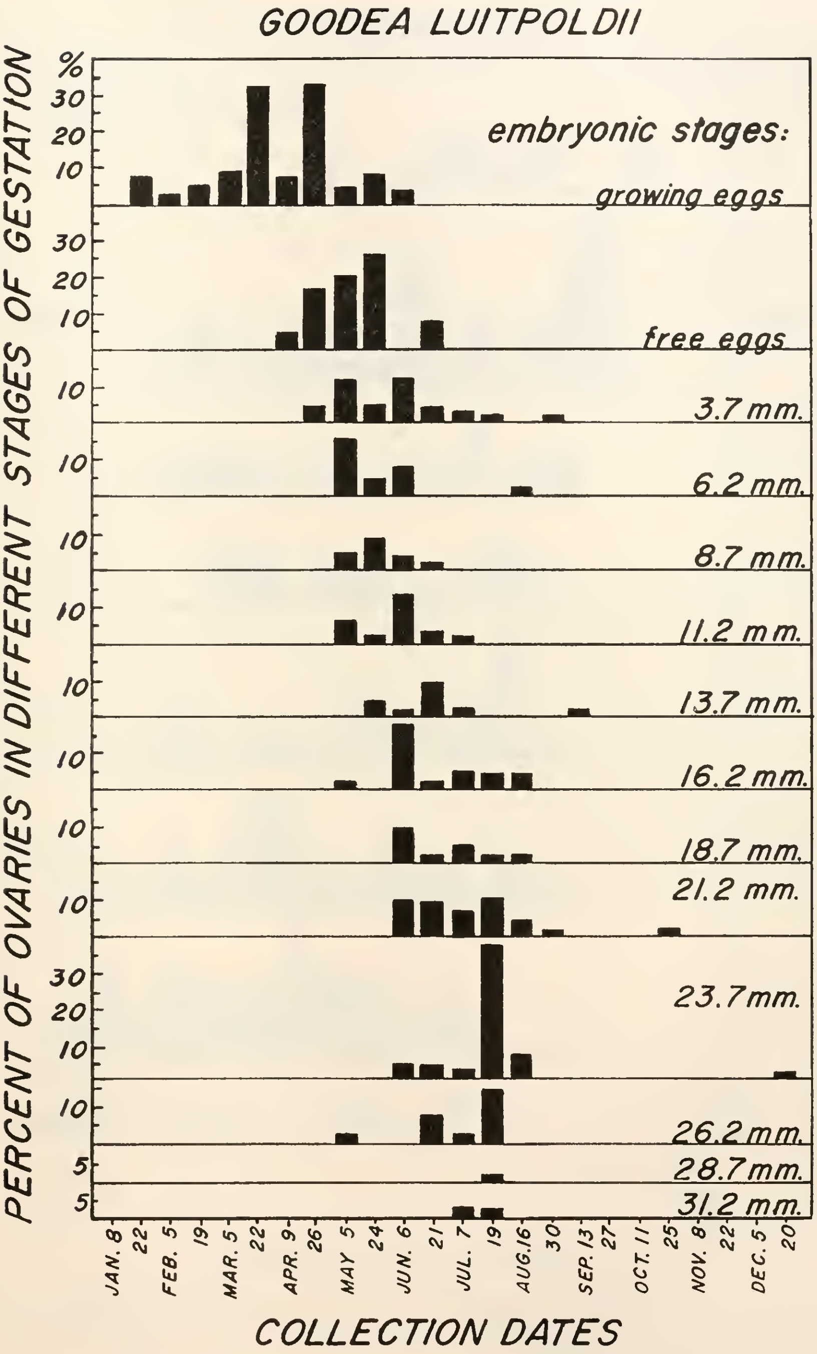

FIGURE 3. The appearance throughout the year of ovaries of *Goodea luitpoldii* with embryos in different stages of development. For any one day, the bars represent the percentage of ovaries in each stage of gestation.

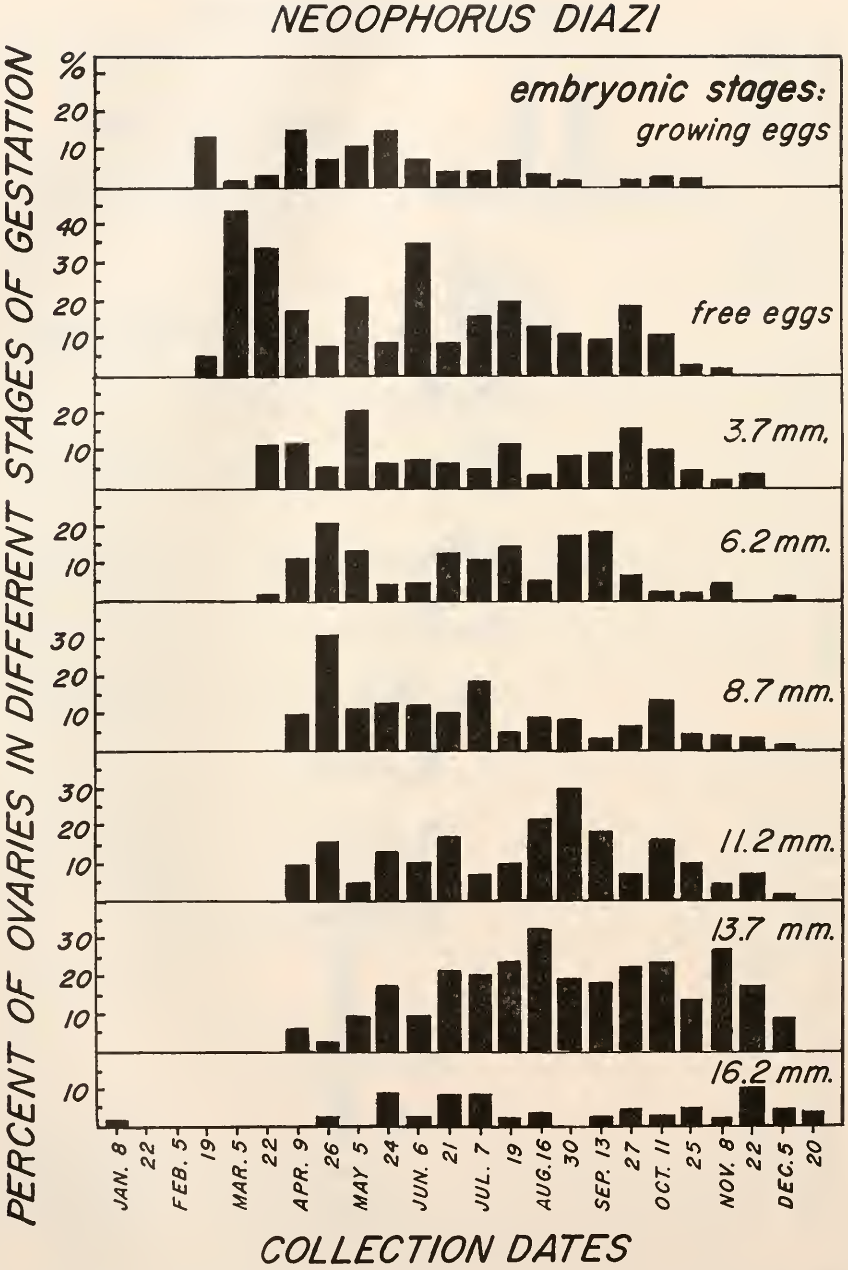

FIGURE 4. The appearance throughout the year of ovaries of *Neoophorus diazi* with embryos in different stages of development. For any one day, the bars represent the percentage of ovaries in each stage of gestation.

to distinguish between post-partum and resting ovaries. The appearance of post-partum ovaries in *Goodea* was the same as in *Alloophorus*. In *Neoophorus*, these ovaries appeared throughout most of the year.

Characteristics of the Gestation Cycle

Length of cycle

Estimates of the length of the gestation cycle in the three species can only be suggestive. In *Alloophorus* there was approximately a two-month period between the first appearance of free eggs (April 9) and the first appearance of post-partum ovaries (June 21). Similarly, the last collection date of free eggs was June 6 and the last appearance of embryos of maximum size was August 16, a period slightly in excess of two months. Similar calculations can be made for *Goodea* and *Neoophorus* from the data. From this it follows that the length of gestation is approximately 60–75 days. This admittedly is only an approximation of the length of gestation but the evidence appears sound.

Number of broods per year

The evidence is conclusive that in *Alloophorus* and *Goodea* there is but one brood per season. In *Alloophorus*, free eggs disappeared from collections even before the first young were born; hence even the females with the first broods could not start a second brood. In *Goodea*, the evidence is similar. In *Neoophorus*, the conditions are quite different. Young were born continuously between April and January; there was no good evidence of periodicity of any kind. Thus the cycle in *Neoophorus* could be a single brood with females starting at different times or the cycle could be a multiple one. If gestation takes approximately two months, these females could undergo at least three broods in one season, depending on the length of the brood interval. The fact that young are born over such a prolonged period during the year explains the difference in the curves for the immature and post-partum ovaries for *Neoophorus* (Fig. 1) as compared to the other two species.

Brood size

Estimates of brood size in the three species are based on sample counts made during different periods of development in the three species. In *Alloophorus* an average of 23.7 young per ovary was counted in a total of 50 ovaries containing 1186 young. In *Goodea*, there were 860 young in 44 ovaries for an average of 19.1 young. In *Neoophorus*, 193 ovaries with 7677 young gave an average of 39.9 young per ovary.

Brood uniformity

All embryos in any one ovary are essentially of the same size. Measurements of all embryos in many ovaries indicated clearly that embryos seldom differed more than 2 to 3 mm. in total length in any one ovary. This uniformity of development is true for all three species.

Abnormal young or runts were very scarce. In *Neoophorus*, runts comprised only 0.49% of a total of 7677 sample embryos; in *Alloophorus* there were 0.42% in 1186 embryos; and in *Goodea* there were only 0.11% in 860 embryos.

Life span of females

There is a general belief among the fishermen that females normally die after reproducing. If this were true, all or most females caught early in the spring would tend to be of minimum length; collections do not support this belief. Measurements of more than 500 females in the three species indicated that fish caught in the spring showed a size range typical for females of each species. However, by using a net with finer mesh unusually small specimens were also caught; these had the following measurements: *Alloophorus,* 60–90 mm.; *Goodea,* 75–85 mm.; and *Neoophorus* 50 mm. or less. The commercial fishermen normally do not keep these small specimens; they form a population with a normal distribution curve at a smaller size-range than that for normal adults. This was true for *Alloophorus* and *Goodea.* It is suggested here that these are one-year-old specimens and that they probably attain maturity during the second breeding season following the year of their birth. Plotting the lengths of all females collected shows a definite bimodal curve; the two peaks presumably represent the two populations, the one-year-old specimens and the normal adults. *Neoophorus,* on the other hand, does not show this condition. A comparable curve for this species is a single but skewed curve. It is likely that these young attain maturity during the breeding season immediately following their birth; consequently, one-year-old young merge into the size-range of the adults. In this species, overlap in size between one-year-old specimens and normal adults is due in part to the extended breeding period of this species.

Age of female and onset of reproductive activity

Making use of collections involving 513 females of the three species, information was obtained concerning the relationship between age and size of the female and brood production. There is no question that the larger females have the larger gonads; this is evident in Table I:

TABLE I

The relationship between size of female and size of ovary in 56 Alloophorus females collected on May 28, 1956

Size of female	Number of females	Average size of ovaries (length × diameter)
90 mm.	8	25.0 × 9.6 mm.
95	12	27.2 × 10.1
100	12	29.0 × 12.0
105	9	37.0 × 15.5
110	8	32.5 × 14.0
115	4	43.5 × 15.2
120 and over	3	43.0 × 19.3

There is a definite correlation between the diameter of an ovary and the size of the female. It should be noted that at this time of the year most females were in some stage of gestation. From these same figures it was determined that the largest young were found in the largest females. This can only mean that the largest females started their reproductive cycle earlier in the season. Except for small variations, the collections for *Goodea* and *Alloophorus* all confirmed the data given above; the May 28 collection is representative. In *Neoophorus,* however, such a progressive relationship does not exist. This can be interpreted only in the light of the extended, presumably multiple and non-rhythmic reproductive cycle of this species. Since there is no evidence of synchrony in the cycle of this species, it means that on any given day any two females of similar size can be in different stages of reproduction. Under these conditions, there can be no consistent relationship between size of female and size of developing embryos.

DISCUSSION

Both *Alloophorus* and *Goodea* have a short reproductive cycle with but one brood per year. In these two species the young were born only during a period of two months; the females were inactive during the rest of the year. It is likely, however, that *Neoophorus* has multiple broods during the breeding season, although conclusive evidence is not indicated by the data. In *Neoophorus* the breeding season extended over a period of 8 to 9 months of the year; this characteristic clearly distinguished *Neoophorus* from the other two species. In this respect the *Neoophorus* cycle approaches that of *Brachyrhaphis episcopi* in which breeding occurs throughout the year (Turner, 1938b). This extensive cycle is not surprising since *Brachyrhaphis* inhabits the area of Barro Colorado Island in the Panama Canal Zone where the tropical conditions are favorable to prolonged periods of breeding. In a sense, the multiple cycle of *Neoophorus* and the single cycles of *Alloophorus* and *Goodea* are antithetic. All forms live in the same lake (Pátzcuaro) and are subject to common physical factors. The long daylight factor at Pátzcuaro, which is in a tropical latitude, could lead to the long breeding cycle of *Neoophorus* but it has not had a similar effect on *Alloophorus* and *Goodea*. On the other hand, Pátzcuaro is at a high altitude (over 7000 feet) and the weather is cool to chilly even in the summer. This temperature factor could be conducive to the single broods in the two larger species but apparently has little effect on *Neoophorus*. Ecological factors in the lake could play a role, since there is some evidence of ecological segregation of species, but the lake is too shallow and probably too homogeneous to provide great ecological differences. In the final analysis, it appears that genetic differences between the three species must perforce play an important role in determining the different reproductive cycles. Among other goodeids, it is known that multiple cycles exist in *Neotoca bilineata* (Mendoza, 1939) and *Xenotoca eiseni* (unpublished data). Among fresh-water viviparous forms, multiple broods are common; this condition is found in forms such as *Heterandria formosa* (Seal, 1911; Turner, 1937); *Gambusia affinis* (Hildebrand, 1917; Turner, 1937); *Lebistes reticulatus* (Turner, 1937; Purser, 1938); *Anableps anableps* (Turner, 1938a) and many others. The single breeding cycle per year found in *Alloophorus* and *Goodea* is more commonly found in marine viviparous teleosts such as *Zoarces viviparus* (Stuhlmann, 1887; Wallace, 1903) and *Cymatogaster aggregatus* (Eigenmann, 1892). It has not been reported in any other goodeid.

In the three species, eggs undergo fertilization immediately before or after escape of the egg from the follicle, since all cleavage and later stages are found only in the ovarian lumen. This condition has been reported for goodeids in general (Turner, 1933), *Neotoca bilineata* (Mendoza, 1941), and it also exists in *Xenotoca eiseni* (unpublished data). Similar early evacuation of the egg from the follicle has been well established for species such as *Cymatogaster aggregatus* (Eigenmann, 1892;

Turner, 1947), *Jenynsia lineata* (Scott, 1928; Turner, 1940b) and others. This condition stands in direct contrast to that found in *Anableps anableps* (Turner, 1938a) and poeciliids in general (Turner, 1947), for fertilization and most or all development takes place in a follicle within the ovarian tissues. The young escape from the follicle only shortly before birth.

Insemination and fertilization occur in rapid succession in the three goodeid species, each brood requiring a separate insemination. Sperm have been observed in the ovaries only about time of fertilization. Although breeding occurs only over a short period of time in the two species, the males of all three species show abundant sperm in the testes during the entire year. In *Neotoca bilineata* (Mendoza, 1941) each of the multiple broods also requires a separate insemination. Furthermore, the phenomenon of sperm storage within the female genital tract does not occur in any goodeid. Stored sperm are believed to permit fertilization of successive broods without necessity for further contact between male and female. The phenomenon of sperm storage and successive fertilization of two or more broods without need for separate inseminations has been described for many viviparous teleosts, such as *Jenynsia lineata* (Scott, 1928; Turner, 1957), *Cymatogaster aggregatus* (Eigenmann, 1892), *Gambusia affinis* (Hildebrand, 1917), *Xiphophorus helleri* (van Oordt, 1928) and others.

Another phenomenon which is absent in goodeids but is very common in poeciliids is the phenomenon of superfetation, a condition in which two or more broods at different stages of development occupy an ovary at the same time. Examples among poeciliids that demonstrate an extreme form of superfetation are *Aulophallus* and *Poeciliopsis* (Turner, 1937), in which as many as nine overlapping broods occur at one time; other poeciliids show varying degrees of superfetation. Failure to achieve superfetation among goodeids is due, in part, to the failure of eggs to grow to maximum size before expulsion of a brood and, in part, to the absence of sperm storage. These two conditions normally occur in many poeciliids and are requisites for the occurrence of superfetation. In contrast to the writer's observations, Turner (1940a) states that he has seen aberrant or unsuccessful examples of superfetation in goodeids such as *Xenoophorus erro, Chapalichthys encaustus, Skiffia variegata* and others, because he has seen sperm and growing oocytes in the ovaries, superimposed on another brood. The possibility certainly exists that occasional eggs may grow, be fertilized, and start development during gestation. In the goodeids studied by the writer, all abnormal embryos observed were so scarce and so close to the stage of development of the current brood that they were all interpreted as abnormalities rather than as younger embryos superimposed on the normal brood.

In *Alloophorus* and *Goodea,* broods average around 20 young but fluctuate under 50. In contrast, broods in *Neoophorus* average about 40 young but may on occasion exceed 100. These brood sizes compare favorably with those in *Xenotoca eiseni* (unpublished data); *Neotoca bilineata* (Mendoza, 1939) has much smaller broods, averaging only six to ten young. Broods numbering under 50 young are very common among viviparous fresh-water fishes. For example, *Gambusia affinis* (Kuntz, 1913) has 40 to 63 young per brood, *Jenynsia lineata* (Scott, 1928) has 10 to 40 young, and there are 30 to 40 young per brood in *Xiphophorus maculatus* (formerly *Platypoecilus maculatus*) (Tavolga and Rugh, 1947; Tavolga, 1949), etc.

In the goodeids studied, the larger and older females have larger broods and, although younger females do have smaller broods, the difference in brood size is not great. This condition is also true in *Neotoca bilineata* (Mendoza, 1939) and *Xenotoca eiseni* (unpublished data) although in these two forms the size of broods in the younger females is markedly smaller. This discrepancy of brood size between younger and older females is very common among other viviparous teleosts, such as *Cymatogaster aggregatus* (Eigenmann, 1892), *Gambusia affinis* (Hildebrand, 1917) *Anableps anableps* (Turner, 1938a) and others.

The occurrence of much uniformity of development among the young in any one ovary is not surprising since this is a common phenomenon. Specific reference to this condition has been reported for viviparous teleosts, such as *Neotoca bilineata* (Mendoza 1941), *Xiphophorus helleri* (Weyenbergh, 1875), *Cymatogaster aggregatus* (Eigenmann, 1892), *Mollienisia latipinna* (Turner, 1937), *Anableps anableps* (Turner, 1938a) and others.

Another impressive factor was the occurrence of very few abnormal embryos during embryonic development. It is likely that if fertilization is successful, the majority of the embryos will continue through development. Reason for this belief rests on the fact that the free egg counts for both *Alloophorus* and *Goodea* agreed well with the average size of broods. In *Neoophorus,* however, there is a greater disparity between the number of free eggs and the number of young in a brood. Even in this species, however, once the embryos start development mortality appears to be very low. The writer's observations do not agree with Turner's generalization that in the Jenynsiidae and Goodeidae many more eggs are fertilized than survive till birth (Turner, 1938a). It is important to note that Turner's observations were not based on the three species in this study.

Finally, the assumption that *Alloophorus* and *Goodea* take two years to mature appears to be unusual among viviparous teleosts. *Zoarces viviparus* (Wallace, 1903) is one of the few described as maturing at the end of the second year. Species such as *Cymatogaster aggregatus* (Eigenmann, 1892) and *Jenynsia lineata* (Turner, 1940b) are said to mature by the following season. It is suspected but cannot be proven that *Neoophorus diazi* matures by the following year. In other goodeids, such as *Neotoca bilineata* (Mendoza, 1939), the young mature within the same breeding season. The length of time necessary for maturation probably is related to sheer physical size of adults, along with pertinent ecological and physiological factors, since *Alloophorus* and *Goodea* are larger than typical poeciliids and apparently resemble *Zoarces viviparus,* another large species (130–300 mm.), in taking two years to mature.

Although some factors in the reproductive cycles of these three species have been demonstrated clearly by the collection of field specimens, it is also evident that some properties of the cycles, such as the actual length of gestation and the single or multiple nature of the *Neoophorus* reproduction cycle, will have to be determined either by tagged specimens in the field or by a laboratory-controlled study.

SUMMARY

1. The reproductive cycles were determined for three goodeids: *Alloophorus robustus, Goodea luitpoldii,* and *Neoophorus diazi.* The study is based on a year-long series of collections in the field; over 3000 females were examined.

2. *Alloophorus* and *Goodea* are shown to have a single cycle; young are born from June through August. *Neoophorus* probably has a multiple cycle and young are born continuously from April through January or February of the next year.

3. Brood size varies as follows: there are approximately 20 embryos per brood in both *Goodea* and *Alloophorus* but the average is about 40 in *Neoophorus*. Younger females have smaller broods although the difference is small.

4. Eggs are discharged from the follicle about time of fertilization and undergo all development within the ovarian lumen. On birth, young are able to swim actively.

5. There is no evidence of sperm storage or superfetation.

6. Embryos in any one brood exhibit much uniformity of size.

7. Abnormal development of embryos is at a minimum; runts constituted less than 1% of all embryos examined.

8. *Neoophorus* is believed to mature in one year whereas *Alloophorus* and *Goodea* are thought to take two years to develop to sexual maturity.

9. Major differences in the reproductive cycle between the three goodeids are believed to be primarily genetic in character.

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AN ANALYSIS OF THE INITIAL REACTION IN THE SEQUENCE RESULTING IN HOMOLOGOUS SPLENOMEGALY IN THE CHICK EMBRYO

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The splenomegaly induced in the chick embryo by chorioallantoic or intracoelomic grafts of homologous adult spleen, or by intravenous inoculations of homologous spleen cells (Danchakoff, 1916; Murphy, 1916; Willier, 1924; Ebert, 1951; Simonsen, 1957; reviewed Ebert, 1958, 1959b) is thought to be the consequence of at least two sequential reactions, an initial graft-versus-host reaction (DeLanney and Ebert, in Ebert, 1957; Ebert and DeLanney, 1960; Simonsen, 1957; see also Billingham and Brent, 1957) followed by a tissue-specific growth reaction, granulocytic proliferation probably being stimulated by products resulting from the partial necrosis produced by the initial immune reaction (Ebert, 1951, 1954; DeLanney, Ebert, Coffman and Mun, 1962; see also Weiss, 1960). Evidence has been advanced also for the involvement of a third process, *i.e.*, a host-versus-graft reaction (Ebert and DeLanney, 1960; Ebert, 1961b); *cf.* Warner and Burnet, 1961), but it is not clear to what extent this reaction contributes to the splenomegaly. It is pertinent to inquire whether these processes can be separated experimentally.

The graft-versus-host phenomenon is but one manifestation of the familiar homograft reaction that leads to the rejection of tissue grafts; in several species, both cold- and warm-blooded, it has been shown to be a consistent and reproducible immunological reaction (Ebert and DeLanney, 1960). The immunological character of this first, destructive phase is widely accepted, being dictated by several lines of evidence.

(1) Recent findings in experiments using grafts of spleen from inbred lines of fowls have demonstrated that interstrain grafts produce a larger effect than intrastrain grafts, a finding to be expected if the reaction were an immunological one. Additional findings to be advanced here agree with those reported by Cock and Simonsen (1958), Mun, Kosin and Sato (1959), and Jaffe and Payne (1961) who used inbred strains of white Leghorn chickens.

(2) X-irradiation of a graft of adult chicken spleen removes its ability to affect the homologous organ of the embryo. According to Mun, Kosin and Sato (1959), after irradiation at low doses, splenic grafts retain their effectiveness; at moderate doses, a significant decrease in effectiveness is observed, and at high doses all activity is lost. Kryukova (1959) also showed that the inoculation of non-irradi-

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ated homologous spleen cells and cells x-irradiated at low doses caused approximately six-fold enlargement of the embryo spleen, yet cells irradiated with moderate doses had little or no effect. Other treatments, *e.g.*, boiling, and freezing and thawing, also remove all activity (Mun, Kosin and Sato, 1959).

(3) The effectiveness of a tissue in producing the reaction varies directly with its content of immunologically competent cells; hence, it appears consistent to state that the reaction is tissue-specific in the limited sense that the specificity reflects the proportion of competent cells a tissue contains. It may be premature to attempt to relate this specificity to a specific cell type, although Terasaki (1959) believes the large lymphocyte to be the common denominator. Whatever the nature of the cell (or cells) concerned, it is known that plasma cells, indicators of immune reactions, may be found at the terminal stages of the reaction (Mori, in Ebert, 1961a). All of the following tissues are effective: bone marrow, liver, spleen, and thymus (Danchakoff, 1918; Willier, 1924; Ebert, 1951). Moreover, Van Alten and Fennell (1959) and Billingham and Silvers (1959), respectively, have shown that a graft of either small intestine or skin also may affect the host's spleen. Although Ebert's earlier (1954, 1959b) observations of quantitative differences in effectiveness were based soundly, his generalization that one might expect a hierarchy of decreasing effectiveness, *e.g.*, spleen, thymus, liver, is difficult to establish. Solomon (1961) was unable to observe such differences and argued that the ultimate extent of the splenomegaly is related directly to the number of competent cells in the graft; certainly this figure will vary, although it is reasonable to assume that generally, spleen and thymus will have a larger number of such cells per unit volume or weight than will other tissues.

The reaction is class-specific; grafts of spleen of other avian species, such as duck, turkey, and pheasant, produce some effect but never as much as homologous spleen; rat, mouse and guinea pig spleens are completely ineffective in the chick (Ebert, 1951, 1954; Mun, Kosin and Sato, 1959). Presumably, the ineffectiveness of mammalian cells is a consequence of their failure to survive the rigors of the foreign environment long enough to produce an immune response. This explanation is being tested experimentally. If it proves to be correct, then mammalian immune mechanisms would appear to be unusually sensitive to the avian environment, for there is evidence for the survival of other kinds of mammalian cells in the chick embryo (*cf.* Clarkson and Karnofsky, in Ebert and DeLanney, 1960, p. 97).

(4) Finally, the ability of splenic grafts to affect the host varies with the age of the donor; grafts of spleen from embryonic donors have little or no effect, the effectiveness of grafts increasing when they are taken from progressively older donors up to several months after hatching. Additional data are presented herein, supplementing the comprehensive recent account by Solomon (1961). Although there are unexplained exceptions to the rule (*cf.* Ebert, 1951; Solomon, 1961, pp. 359–363), the effectiveness of grafts is related directly to their immunological maturity. Our perspective of the problem of maturation of the immune response has been broadened by the findings of Makinodan and Peterson (1962) who have observed that the relative antibody-forming capacity of spleen cells of mice varies with age from one week to 29 months. A rapid increase in activity was noted from one week to one month, one less rapid from one to 8 months. A gradual decrease was then observed from the peak at 8 months through an additional 21 months.

Accepting the argument that a part of the splenic enlargement following a graft of adult spleen encompasses immune reactions, we may take up next the site of these reactions. How many cells leave the graft and enter the extraembryonic membranes and the embryo itself? How many donor cells take up residence in the homologous organ of the host? Do they also settle in other organs? It is clear that when suspensions of adult chicken spleen cells or suspensions of adult chicken lymphocytes are administered to the embryo intravenously, or when grafts of adult spleen are made to the chorioallantoic membrane or into the coelom, *some* of the donor cells colonize the organs of the host. The evidence is derived from serial transfer studies by Simonsen (1957) and Ebert and associates (Ebert, 1957; DeLanney, Ebert, Coffman and Mun, 1962). When a graft of adult chicken spleen is made to the coelom of a four-day-old chick embryo, the host's spleen is enlarged four- to five-fold within six days. If fragments of this greatly enlarged ten-day-old embryonic spleen now are transferred to new four-day-old hosts, they elicit a reaction of the same order of magnitude, whereas fragments of spleen from normal ten-day-old embryos are ineffective. After nine successive transfers, the effectiveness of the implant is not reduced markedly below the level attained by the primary graft. Assuredly, then, there is some colonization. But how much, and to what extent do these donor cells proliferate? Simonsen (1957) argued that colonization and proliferation accounted for all the effects of splenic grafts. However, studies by Ebert and associates (summarized by DeLanney, Ebert, Coffman and Mun, 1962) of the cellular nature of the response pointed to the host as the principal source of proliferating cells. Moreover, studies using grafts radioactively labeled, in early experiments with sulfur^{35}, while not decisive, revealed a predominant localization of material in the homologous organ, but precluded a massive transfer of cells (Ebert, 1954, 1959b). Biggs and Payne (1959) have presented significant findings in a study in which they identified proliferating donor cells in chick embryos injected with adult chicken blood. In the chicken the fifth largest chromosome is paired in the male, unpaired in the female. Cockerel blood was injected into fourteen-day-old embryos which were sacrificed at day eighteen. In enlarged spleens taken from female embryos, male chromosomes could be identified, proving the localization of some donor cells. The relatively high number of dividing female cells, however, suggested to Biggs and Payne that an appreciable component of the splenic enlargement is provided by cells of the host. The evidence available, therefore, suggests that following the intravenous injection of blood or spleen cell suspensions, some donor cells colonize the host's spleen. Moreover, such colonization need not result invariably in splenic enlargement, which may result in whole or in large part from proliferation of cells of the host.

The fact that splenomegaly is not evoked by noncompetent homologous donor cells or with competent isologous cells forces the conclusion that the *proliferation of cells of the host is a secondary consequence of a primary immune reaction.* The nature of this secondary reaction must be the principal target of future investigations. In beginning such a study, it became clear that more information was needed on the extent of colonization and maintenance of donor cells in the several tissues of the host.

It is the objective of this report to present findings bearing on that question; these findings bear importantly also on another question, namely, the ability of the embryonic environment to support an immune reaction. Preliminary accounts of some of these findings have been published (Mun, Errico and Ebert, 1961; Ebert, 1961a, 1961b).

MATERIALS AND METHODS

Non-inbred white Leghorn chickens and eggs were supplied by Elder Farms, Hyde, Maryland. Hybrid white Leghorn chickens and eggs were obtained from Truslow Hatchery, Chestertown, Maryland. Chickens and eggs from two inbred lines (7 and 15) with coefficients of inbreeding of greater than 95% were supplied by B. Winton, Director of the Regional Poultry Research Laboratory, East Lansing, Michigan. New Hampshire red eggs were purchased from Red Gate Farm, Newport, New Hampshire. Eggs were incubated in a Jamesway incubator at 37.5 to 38.0° C.

The aseptic grafting technique employed was that described by Willier (1924; see also Hamburger, 1960). A quadrilateral window (1 × 1 cm.) was cut in the shell with a fine-toothed hacksaw blade. The shell membrane was punctured and reflected, and a fragment of tissue measuring approximately 1 × 1 × 2 mm., and weighing 5 to 10 mg., was placed on the chorioallantois. The shell membrane and shell were replaced and sealed with paraffin. The eggs were placed in the incubator with the small end down.

The eggs were operated on the ninth or tenth day of incubation. After 7 or 8 additional days of incubation the graft was removed and examined. The size of the area of implantation (length × width) was recorded and the condition of the implantation site was graded as follows: (1) graft enlarged, pink, and larger than the original; (2) graft pink and as large as the original implant; (3) graft brown or green, clearly not incorporated and smaller than the original or grafted tissue. Rarely a graft in category (3) produces a response, but the fact that a reaction did occur occasionally suggests the movement of viable cells from the graft soon after implantation. Spleens of recipient embryos were removed and weighed to the nearest 0.2 mg. The weights of spleens from embryos in group 3 were not included in the tabulations.

RATE OF COLONIZATION IN THE CHORIOALLANTOIC MEMBRANE ADJACENT TO SPLEEN GRAFTS, AND IN THE HOSTS' SPLEENS

DeLanney and Ebert (1959a, 1959b), and DeLanney, Ebert, Coffman and Mun (1962) have followed the cytological changes in the chorioallantois at closely timed intervals after implantation of homologous adult spleen. Immediately after implantation the epithelium of contact thickens; the mesenchyme forms spindle cells and undergoes a shift toward myelogenesis. In the zone of contact between graft and membrane, the chorionic epithelium is eroded, clusters of granulocytes appear, spindle cells gather at the border, and tongues of cells, apparently originating in the graft, invade the membrane. The second set (or third set) chorioallantoic transplantation of fragments of chorioallantois taken from reactive sites surrounding the original first set spleen implant results in intensified reactions. Further evidence of colonization of the membrane is provided by the following experiments, in which fragments of homologous embryonic spleen were placed on the chorioallantois some distance from grafts of homologous adult spleen. After varying intervals, the embryonic grafts were removed, and their ability to produce splenomegaly determined.

Two windows, approximately 1 cm. apart, were cut in the shell. A fragment of adult spleen, kidney, or heart was placed on the chorioallantois through one window and 17-day-old embryo spleen was implanted through the other. In control groups embryonic spleen was implanted in both sites. After 7 additional days of incubation, the adult graft and the embryonic graft were removed, with associated membrane, and implanted on the chorioallantoic membrane of new 10-day-old hosts. After 7 additional days of incubation the hosts' spleens were removed and weighed. Table I shows that an embryonic spleen graft placed adjacent to an adult spleen graft can affect the host's spleen to the same extent as an adult spleen graft. Here, then, is further evidence of movement of cells from the adult spleen graft to a graft of embryonic spleen on the membrane. How rapid is this movement?

TABLE I

Colonization of embryonic spleen grafts adjacent to grafts of adult spleen and other tissues

Donor	No.	Mean weight of host spleen (mg.)	SE_m
Adult spleen + adult spleen	4	47.4	—
Graft of adult spleen	3	39.1	—
Graft of host's spleen	2	118.4	—
Adult spleen + embryo spleen	30	35.9	3.8
Graft of adult spleen	4	38.9	—
Graft of embryo spleen	29	40.3	4.9
Graft of host's spleen	2	36.1	—
Adult kidney + adult kidney	26	17.2	1.7
Graft of adult kidney	14	19.9	3.2
Graft of host's spleen	4	21.7	—
Adult kidney + embryo spleen	22	18.9	2.1
Graft of adult kidney	14	12.1	4.2
Graft of embryo spleen	19	38.8	6.6
Graft of host's spleen	4	42.3	—
Adult heart + adult heart	9	12.2	0.8
Graft of adult heart	6	18.4	4.9
Graft of host's spleen	6	18.7	4.4
Adult heart + embryo spleen	12	14.9	1.2
Graft of adult heart	3	14.2	—
Graft of embryo spleen	10	16.6	1.6
Graft of host's spleen	9	20.5	3.5
Embryo spleen + embryo spleen	10	13.7	0.9
Graft of embryo spleen	8	12.4	1.0
Graft of host's spleen	3	14.6	—

Homologous spleen and homologous embryonic spleen were implanted approximately 1 cm. apart as described above. After 2, 3, 5, 6, or 7 additional days of incubation both grafts and the host's spleen were removed and transferred to new

10-day hosts. Table II shows that, as early as *two* days after implantation, both the embryonic spleen graft and the host's spleen, neither of which show any enlargement at this time (*cf.* DeLanney, Ebert, Coffman and Mun, 1962), are capable of affecting the spleen after serial transfer.

COLONIZATION OF ADJACENT MEMBRANE BY ADULT SPLEEN CELLS LABELED WITH TRITIATED THYMIDINE

The suggestion that the use of cells labeled with tritiated thymidine might aid in resolving the question of migration of adult chicken spleen cells from grafts to the hosts' membranes and spleens was advanced by Ebert (1959b). However, the principal limitation of the method, the dilution of label in rapidly dividing cells, is critical, and in the opinion of the writers the technique is less reliable than the cytological method, *i.e.*, recognizing donor cells by sex chromosome differences (Biggs and Payne, 1959; Ohno, 1961). However, the following experiments do contribute further to our knowledge of the migration of cells into the adjacent membranes.

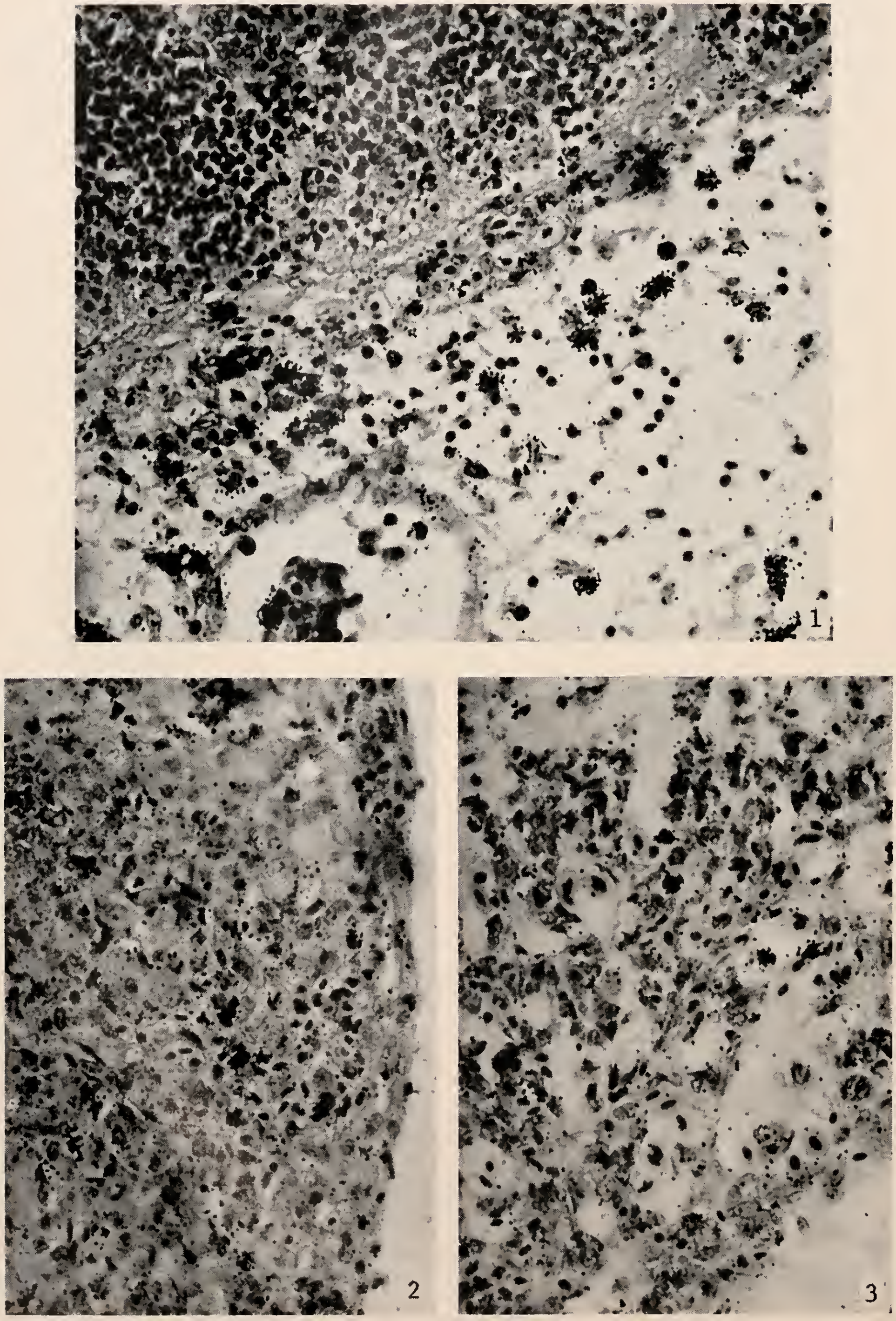
TABLE II

Rate of colonization of host embryo's spleen (HS) and embryonic spleen graft (GES) adjacent to adult chicken spleen graft (GAS) on the chorioallantois

Donor	2–3 days			5 days			7–8 days		
	No.	Mean weight of host's spleen (mg.)	SE_m	No.	Mean weight of host's spleen (mg.)	SE_m	No.	Mean weight of host's spleen (mg.)	SE_m
AS + ES									
GAS	13	21.6	6.8	2	47.8	—	4	38.9	—
GES	21	21.8	3.4	5	21.8	6.3	29	40.3	4.9
HS	14	23.1	5.1	6	17.5	2.0	30	35.9	3.8
ES + ES									
GES	6	10.8	1.2				8	12.4	1.0
HS	4	11.4	—				3	14.6	—

Nineteen experiments were conducted using labeled and non-labeled donor material. The results of three experiments (XI, XIII, XIIIa) will be considered here.

In experiment XI, the donor tissues were labeled by injecting into the wing veins of adult white Leghorn chickens 2 millicuries of tritiated thymidine in two doses, 48 and 24 hours before sacrificing. In experiments XIII and XIIIa, labeled tissue from enlarged embryonic spleen was used as donor tissue. The cells were labeled by injecting 15 to 25 microcuries of tritiated thymidine into the yolk sacs of 9-day-old chick embryos. Twenty-four hours later, a piece of unlabeled adult chicken spleen was implanted on the chorioallantois of each embryo (Fig. 1). In control series, a piece of homologous embryo spleen was implanted instead of adult spleen. After 7 or 8 additional days of incubation the enlarged



Figures 1–3.

and labeled host spleens were removed, cut into small pieces, and implanted in 9-day-old chick embryos.

The latter approach resulted in at least a doubling of the percentage of labeled donor spleen cells. The enlarged embryo spleen also elicited a greater increase in the size of the host spleen than grafts of adult chicken spleen. Large white nodules and lesions were observed more frequently in foot and head regions.

At recovery a number of tissues, including the membrane containing the graft, the host's spleen, and a sample of blood, were obtained from each embryo. Representative whole embryos also were fixed in Bouin's fluid.

The labeled cells were detected by autoradiography. The host's spleen and membrane containing the graft were sectioned at 5 microns, and stained with Mayer's hematoxylin and eosin. The slides were then coated with Kodak NTB-3 photographic emulsion, following in general the procedures developed by Messier and LeBlond (1957) and Everett and Simmons (1953). Approximately three drops of a 50% emulsion kept at 40° C. were smeared on the surface of the warmed glass slide with a wet brush. The smear was slowly rocked to remove the brush marks. Excess emulsion was shaken off, and the slide was permitted to dry in a near-vertical position. The coated slides were kept in the refrigerator (4 to 10° C.) and developed in D72 or D19 (Kodak) after 14 days.

In each experiment, more than 30 embryos received grafts of adult chicken spleen or enlarged embryo spleen on the ninth day of incubation. An equal number received labeled embryonic spleen, labeled adult chicken kidney, or irradiated and labeled adult spleen grafts. The different categories of active and inactive, as well as labeled and nonlabeled donor tissue, also were combined on the membrane of the same host. A small number was untreated or received three drops of saline.

A number of embryos from each group, selected at random, were recovered at postoperative days 1, 2, 3, and 7. Another group, sufficient in numbers to ascertain the degree of enlargement of the host's spleen elicited by the donor material, was recovered on the eighth day after the operation (Table III).

In embryos in which donor spleen tissues containing distinctly labeled cells (see Figure 2) were grafted, labeled cells were still detected in significant numbers in the graft as well as the adjacent membranes up to the fifth postoperative day (Fig. 3). By the eighth postoperative day, however, labeled cells were not readily detected in the graft and adjacent tissues. Preliminary examination of spleens from host embryos, which contained distinctly labeled donor spleen cells in the CAM graft, revealed few or no distinctly labeled cells. Quantitative evaluation of the autoradiograms is in progress; however, these preliminary observations do not suggest a direct large scale migration of donor cells from the CAM graft to the host's spleen.

FIGURE 1. Chorioallantoic membrane showing the edge of a graft of adult chicken spleen. Chick embryo injected via the yolk sac with 25 microcuries of tritiated thymidine, and the unlabeled graft implanted on the ninth day of incubation; recovered 24 hours later. ×500.

FIGURE 2. Section of spleen from an embryo which received 25 microcuries of tritiated thymidine and a graft of adult chicken spleen on the ninth day of incubation; recovered after 8 days. ×500.

FIGURE 3. Section of chorioallantoic membrane of a 13-day embryo containing cells of a labeled graft of "second-set" embryonic spleen, implanted 4 days earlier. ×500.

TABLE III

Mean weight of host's spleen after chorioallantoic grafting of adult and embryonic spleen

Expt. (see text)	Day recovered post-operative	Donor	No.	Average weight of host spleen	SE_m
XI	8	Adult chicken spleen	5	34.0	2.6***
		Saline control	7	10.5	0.6
XIII	8	Enlarged embryo spleen	10	86.2	8.3***
		Saline	5	12.4	1.4
$XIII_a$	8	Enlarged embryo spleen	7	29.4	4.5**
		Saline	4	8.8	1.0

** Significant at the .01 level.
*** Significant at the .001 level (*t* test).

COLONIZATION IN OTHER ORGANS

It might be expected that competent cells from a graft might be lodged in all tissues of the embryo's body, to some extent, possibly to the same extent to which the adult tissues normally contain lymphoid and other reticulo-endothelial elements. This expectation is realized.

Adult chicken spleen was implanted on the chorioallantois of 9-day host embryos. After 7 more days of incubation the host's spleen, liver, heart, and kidney were removed and implanted on membranes of new 9-day recipients. Table IV shows an increase in the weights of such secondary hosts' spleens receiving grafts of "second set" spleen, liver, heart, and kidney, to approximately the same extent as that elicited by the corresponding adult organs.

Thus there is a transfer of competent cells not only to the host's spleen but to other organs as well.

SERIAL PROPAGATION IN CHICK EMBRYOS OF EMBRYONIC SPLEEN CELLS FROM NON-INBRED AND INBRED CHICKENS

The graft-versus-host reaction has provided unequivocal evidence that, beginning as early as the fourth day of development, the chick embryo provides an

TABLE IV

Effect of grafts of fragments of adult organs and organs from embryos stimulated by seven days' exposure to adult spleen grafts on the weight of the host embryo's spleen

Nature of grafts	Spleen weight (mg.) following grafts of			
	Spleen	Liver	Heart	Kidney
Adult organs	47.4 (4)*	10.8 (7)	18.7 (6)	17.2 (26)
Host embryo organs after grafting of adult spleen	42.7 (6)	18.0 (6)	12.1 (7)	23.6 (6)
Embryonic organs	14.6 (3)	11.4 (5)	12.3	8.0
Host embryo organs after implantation of embryonic spleen on the membrane		16.0 (3)	11.9 (3)	12.4 (3)

* Figures in parentheses indicate number of cases.

environment favorable for at least one class of immune reactions, those of transplantation immunity (Ebert, 1961b). Competent cells retain their competence in the embryonic environment. But would incompetent embryonic spleen cells which were maintained in an embryonic environment for long periods of time ever reach a stage of functional maturity? Or, to put the question in more practical terms, if homologous embryonic cells were transferred from the graft to the host's spleen where they proliferated, after several transfers the donor embryo cells thus maintained in this embryonic environment should eventually attain maturity and be able to elicit an enlargement of the host spleen. On the other hand, if the donor embryo cells were incorporated in the host's spleen but did not proliferate, they would be diluted after only a few passages.

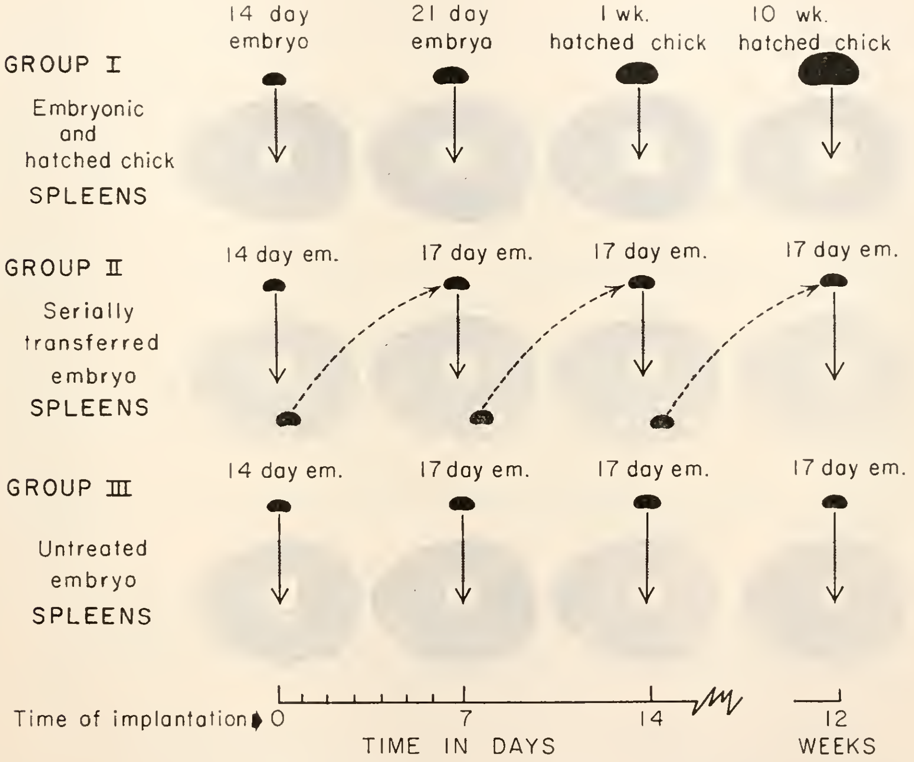

GROUP I
Embryonic and hatched chick SPLEENS
14 day embryo
21 day embryo
1 wk. hatched chick
10 wk. hatched chick
GROUP II
Serially transferred embryo SPLEENS
14 day em.
17 day em.
17 day em.
17 day em.
GROUP III
Untreated embryo SPLEENS
14 day em.
17 day em.
17 day em.
17 day em.
Time of implantation
0
7
14
12
TIME IN DAYS
WEEKS

Figure 4. Serial grafting of homologous embryonic spleen.

Four groups of embryos have been studied: (1) Fragments of homologous spleen from successively older embryos and hatched chicks were grafted to the membranes of 10-day-old chick embryos at weekly intervals. After 7 additional days of incubation, the hosts' spleens were removed and weighed to the nearest 0.2 mg.

(2) A fragment of homologous 14-day embryonic spleen was placed on the membrane of a 10-day host embryo. After 7 additional days of incubation, the host's spleen was removed, weighed, and transferred to another 10-day embryo.

This procedure was repeated for 7 or 11 weeks. The donor spleens were not pooled but were kept separate. Thus, for each of the 20 initial donors 20 separate lines may be traced.

(3) Fourteen- or 17-day-old embryo spleens were placed on the membranes of 10-day host embryos at weekly intervals. After 7 additional days of incubation the hosts' spleens were removed and weighed.

These three groups are illustrated graphically in Figure 4.

(4) The weights of the 14- or 17-day embryo donor spleens before grafting comprise the fourth group. This group is not included in the final tabulation because greater variation in spleen weight was observed following implantation of a piece of embryo spleen. Thus the spleen weights in the third group were better controls for the second group.

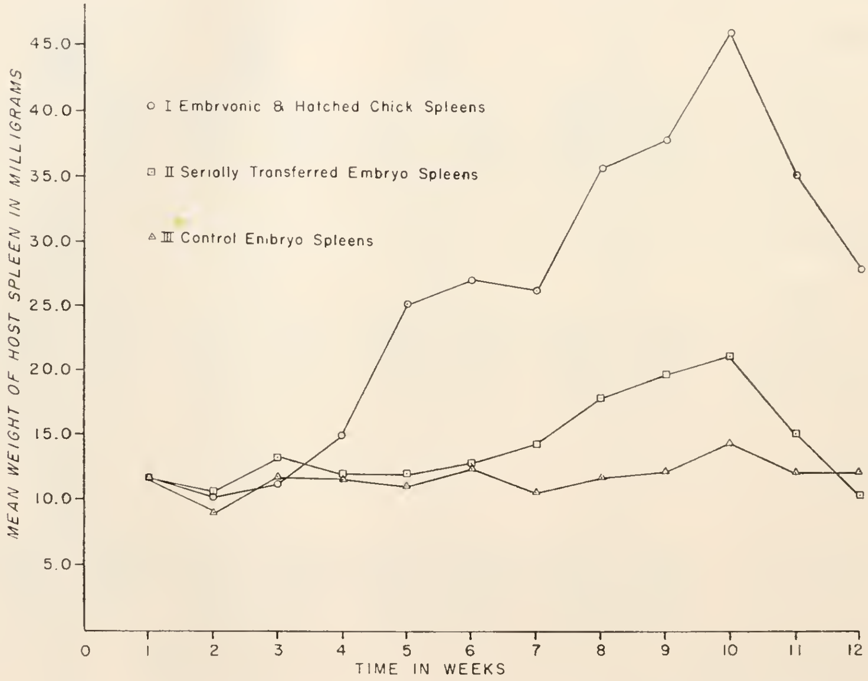


FIGURE 5. Splenomegaly after serial grafting of homologous embryonic spleen.

The first experiment was carried out over an 8-week period. Because an increase in the average weight of the host's spleen in the serial group (2) was observed in the eighth week, the second experiment was carried out over a longer 12-week period.

The results of the two experiments were consistent, hence the data were pooled.

As may be seen in Figure 5, spleen grafts from successively older embryos and hatched chicks after a short lag period of one or two weeks produce a progressively greater enlargement of the host chick embryo spleen. A significant enlargement of the host embryo spleen is produced by spleen from a 3-week-old hatched chick.

These findings are in general agreement with those of Solomon (1961) and the previously unpublished data of DeLanney (cited in Solomon, 1961) who observed an approximate doubling in spleen size following grafts of spleen from 28-day-old juvenile chickens. DeLanney's independent findings are not strictly comparable to those set forth here, the period of exposure (days 7 through 18) and weighing procedure being different, hence they are not included in the tabulation; the data may be obtained from him upon request.

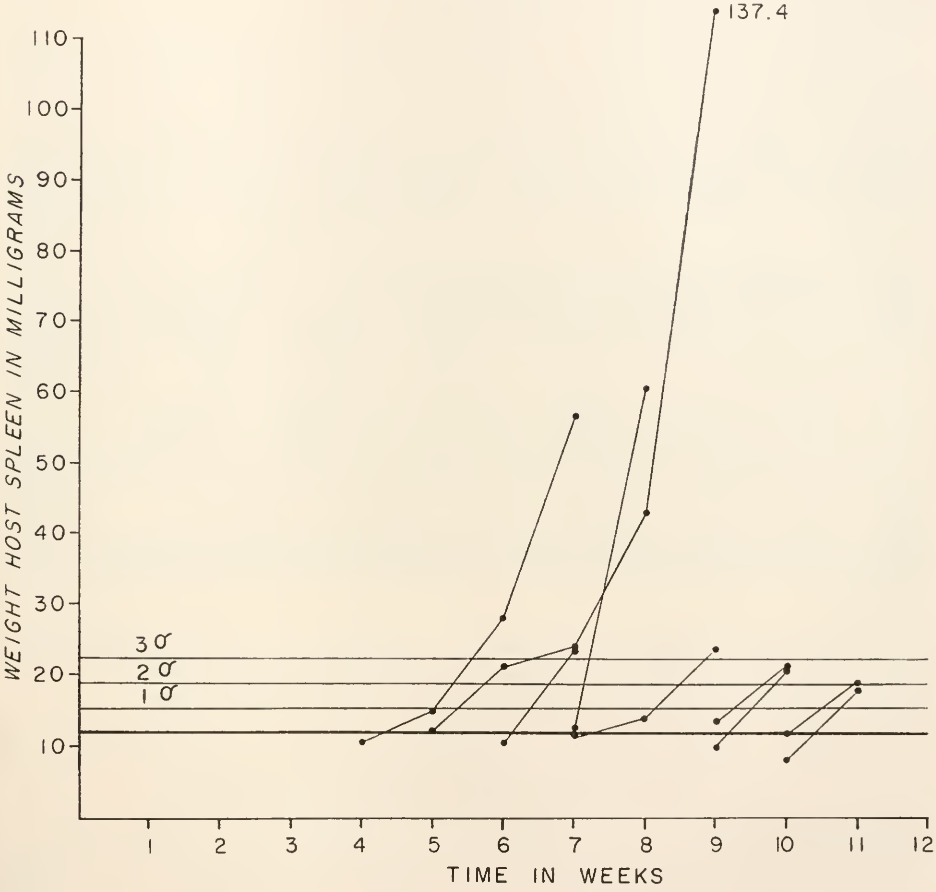


FIGURE 6. Splenomegaly in individual lines after serial grafting of homologous embryonic spleen.

In the second or serial group, after a lag period of 6 to 7 weeks, a distinct increase is observed in the average weight of the host's spleen. However, the differences in the mean weight between the serial group and the third, control, group even at the peak of 8 weeks is significant only at the 5% level as determined by Students "*t*" test.

However, when one follows the changes in spleen weights of subsequent hosts in each of the individual lines in the serial group, a clearer picture emerges. Of the 30 initial donors only 14 lines were successfully transferred for 8 to 12 weeks. Figure 6 shows fragments of 9 of these 14 surviving lines. The lag period of 6 to 8 weeks is not shown. In 4 of these 14 lines, after the lag period, there is a definite progressive increase in the weights of the hosts' spleens. Five other lines show this increase to a lesser extent and 4 lines do not show any change in weight of the hosts' spleens.

The individual weights of many of the host spleens at the peak of the growth phase deviate greatly from the distribution of the control (group 3). The 3 s level (97%) for group 3 is shown (22.3 mg.).

The data show that in 4 out of 14 lines the effects are cumulative, *i.e.*, an increase in spleen weight occurs with each successive transfer. We may interpret these observations as indicating that in these 4 lines, cells of donor origin are transferred from the graft to the host's spleen. It would appear that these embryonic spleen cells proliferate and are maintained in the host embryonic environment. After several transfers, following the pattern of development of the normal chick spleen, the cells mature immunologically. The splenomegaly thus induced is serially propagated.

One may ask next, are the cells which produce this effect truly derived from the *first,* or do they stem from the second, or any of the subsequent transferred spleens? Is a single initial exposure to antigen sufficient to produce splenomegaly in subsequent hosts of a series? At least a tentative answer to this question is obtained by the use of embryos from two inbred lines of chicks.

Mun, Kosin and Sato (1959), using two inbred lines of chickens, found that a greater splenomegaly was obtained when the donor tissue was derived from an adult chicken of the opposite line than from the same line. Cock and Simonsen (1958) made similar observations using injection techniques. It should be possible to determine if a single exposure is sufficient by the following experiment, illustrated diagrammatically. One need only compare the effectiveness of the two series:

(1) A → B → B → B → B → B → B and (2) B → B → B → B → B → B → B,

where A is the donor spleen from one line and B is the other line.

The effects of spleens from two inbred lines were first compared within and between lines. Spleen tissue from one-month-old line 7 and line 15 chickens were implanted, reciprocally and within lines to the membranes of 10-day-old embryos. As shown in Table V there is a striking difference in the reaction of these two lines to line 7 donor spleen but not to line 15 donor spleen. However, the line 7 embryo spleen was affected somewhat more greatly by line 15 donor spleen than by line 7 donor spleen. These differences are significant on the basis of the pooled t test.

The serial experiment as outlined above was then carried out on embryos from these two inbred lines. Four groups of embryos were treated as follows:

(1) A 17-day line 7 embryo spleen was placed on the membrane of a 10-day line 15 embryo. After 7 additional days of incubation, the host's spleen was removed, weighed to the nearest 0.2 mg. and cut in half. One half of the host's spleen was transferred to the membrane of another line 15 embryo. After 7 days the host's spleen was again removed, weighed, and treated in similar fashion. The average weight of the line 15 hosts' spleens forms the first group (I) in Table VI.

TABLE V

Comparison of the ability of spleens from two inbred lines of chickens to affect spleens of embryos from these two lines

Line of adult donor	Line of host embryo	No.	Average weight of host's spleen (mg.)	S.E. of mean
7 (4 donors)	7	28	13.0	0.5
	15	17	75.5	8.4
15 (3 donors)	7	22	29.3	1.8
	15	17	25.2	2.8

(2) The other half of the line 15 embryo spleen was placed on the membrane of a line 7 embryo. After 7 additional days of incubation, the host's spleen was removed and weighed, but not transferred. The average weight of the line 7 hosts' spleens form the second group (II).

(3) In the third group, a 17-day line 15 embryo spleen was grafted to a 10-day embryo from the same line. After 7 additional days of incubation, the host's spleen was removed, weighed, and cut in half, one half being transferred to a new host of the same line. The average weight of the line 15 hosts' spleens forms the third (III) group.

(4) The other half of the line 15 spleen was grafted to a line 7 embryo. After 7 additional days of incubation, the spleen was removed and weighed but not transferred. The average weight of the line 7 hosts' spleens forms the fourth (IV) group.

(5) As further controls, untreated 17-day-old lines 7 and 15 embryo spleens were grafted on line 7 embryos each week. The average weight of the donors' and hosts' spleens formed a fifth (V) group. The data for this group are not included in the table.

As shown in Table VI a significant increase in the weights of the hosts' spleens was not observed in any group after 8 transfers. These results suggest that a

TABLE VI

Mean weight of spleens of four groups of inbred embryos serially transferred at weekly intervals

Week number	Group I	Group II	Group III	Group IV
1	10.2 (18)*		10.1 (18)	
2	11.4 (11)	9.3 (10)	13.9 (13)	10.1 (12)
3	13.3 (15)	10.2 (5)	13.8 (17)	10.7 (9)
4	13.4 (14)	10.5 (10)	13.2 (12)	10.4 (12)
5	13.5 (16)	9.9 (11)	12.4 (15)	10.0 (11)
6	13.8 (14)	11.0 (12)	13.6 (10)	10.0 (8)
7	13.4 (15)	9.4 (12)	14.5 (13)	10.5 (7)
8	14.4 (18)	7.8 (12)	13.5 (17)	9.3 (8)
9	14.9 (15)	8.2 (11)	13.2 (12)	10.8 (11)

* Figures in parentheses indicate number of cases.

single exposure (A → B) was not sufficient to initiate the reaction. In view of the observation that a subsequent increase in weight of the hosts' spleens was obtained in serial transfers of spleens from non-inbred embryos, it must be suggested that the latter effect is cumulative. Homologous cells of different genetic makeup are accumulated gradually in the spleen with each transfer, resulting eventually in the observed reaction.

However, in view of the fact that the homologous hosts and donors involved were all embryonic, why was a mutual immunological tolerance not developed? We were led to inquire then whether "tolerance," as measured by the prevention of splenomegaly, could be induced by the exposure of 10- to 17-day-old chick embryos to grafts of embryonic spleen?

EFFECT OF SPLEENS FROM ADULT NEW HAMPSHIRE RED CHICKENS WHICH HAD RECEIVED CHORIOALLANTOIC GRAFTS OF WHITE LEGHORN EMBRYO SPLEEN ON THE NINTH DAY OF INCUBATION

Terasaki, Cannon and Longmire (1958) injected 0.4 ml. of blood intravenously from 10- to 16-day-old white Leghorn (WL) embryos to New Hampshire red (NH) embryos and vice versa. Two or 15 days after hatching, skin from chicks other than the blood donor, but of the same breed as the blood donor, was grafted. A significant percentage of these homografts survived longer than grafts between control chicks not previously injected with blood. This observation was extended to include interbreed differences by Kulangara, Cannon and Longmire (1959). Tolerance of skin homografts may be obtained by embryonic injection of blood from a breed of chicken other than that of the skin donor. The following series of experiments was designed to answer the question, can embryos of one breed (NH) be made "tolerant" with respect to the ability to affect the spleen of another breed (WL)?

Embryo spleens pooled from five 19-day-old white Leghorn embryos were minced and pipetted on the membranes of 10-day New Hampshire red hosts. The operated eggs were permitted to hatch. On the second, third, and tenth week post-hatching, spleens from treated and untreated chickens were implanted on the membranes of 10-day-old WL hosts. The results are shown in Table VII.

TABLE VII

Effect of spleens from 2-, 3-, and 10-week-old New Hampshire red (NH) chickens grafted with white Leghorn embryo spleens (WL-ES) on the 10th day of incubation

Donor	No.	Mean weight of host's spleen (mg.)	SE_m
2-week-old NH + WL-ES	21	16.1	1.5
NH not treated	24	16.4	3.9
3-week-old NH + WL-ES	24	14.8	1.2
NH not treated	12	16.4	1.9
10-week-old NH + WL-ES	31	32.2	3.7
NH not treated	28	28.2	3.5

The effect on the host spleen of 2- to 3-week-old chicken spleen is not large (see Figure 5) but there does not appear to be any difference between the groups. Spleens from 10-week-old chickens produce a four-fold enlargement. Again, there does not appear to be any difference in the ability of the spleens from treated and untreated chickens to elicit splenomegaly. Under the conditions employed, therefore, tolerance is not induced. Possibly the relative ineffectiveness of the membrane implantation technique, in contrast to intravenous injection, is to be stressed. In any event, there are insufficient grounds here for questioning the idea of interbreed tolerance in chickens.

Reduction in Effectiveness of Adult Spleen Following Pre-immunization

The availability of inbred lines of chickens made it possible to test further the possibility of pre-immunizing adult chickens and producing unusually rapid and severe graft-versus-host reactions. Earlier attempts with non-inbred fowls (Mun, Kosin and Sato, 1959; Van Alten, 1961) had produced anomalous results. The specific question to be answered is the following: will spleens from adult chickens of one line which have rejected skin grafts from another line produce a greater effect in hosts of the donor line than spleens from animals which had not previously rejected such skin grafts?

Skin grafts were performed on 10-day-old hatched chicks from inbred lines (7 and 15), both between and within these two lines. After one month, a great majority of the skin grafts received from the opposite line (homografts) began to disintegrate and slough, leaving large open wounds at the site of the graft. Three chickens (two from line 7 and one from line 15) showing the graft rejection reaction were sacrificed and fragments of their spleens were implanted on the membranes of 10-day-old line 7 and line 15 embryos. As controls, chickens with intact skin grafts from chicks from the same line (isografts), as well as autografts, and untreated chickens from each line were sacrificed at the same time. The results of these chorioallantoic grafts are shown in Table VIII. Spleen implants from chicks showing the graft rejection reaction did not elicit a greater enlargement in the reciprocal line host than spleen implants from the control chicks. In fact, the average weight of the host spleen was somewhat less than that of the control group in both lines.

These observations are similar to those reported by Mun, Kosin and Sato (1959) and Van Alten (1961). In the former experiments, adult chickens were injected intravenously and intraperitoneally with pooled 15- to 19-day-old chick embryo spleens. The spleens from these injected chickens did not produce a greater increase in the size of the host embryo spleen. Instead, the effect of spleen from the injected chickens was consistently and significantly less than that of spleens from non-injected chickens. Terasaki (1959) made similar observations. Donor chicks were immunized by skin grafting or by injection of spleen cells intravenously and intraperitoneally. Neither lymphocytes nor spleen cells from these immunized chickens when injected into embryos isologous with the immunizing tissue produced marked splenic enlargement or earlier deaths.

Simonsen and Jensen (1959) observed a marked graft-versus-host reaction (higher spleen indices) in the hybrid mouse $(C_3H \times AKR)F_1$ host when the

TABLE VIII

Effect of spleens from inbred WL adult chickens which had rejected skin grafts from the opposite line

Line of donor	Treatment of donor and condition of graft	Line 7 host			Line 15 host		
		No.	Mean weight of host's spleen	SE_m	No.	Mean weight of host's spleen	SE_m
7	(B3) rejected skin graft from line 15	5	12.2	13.3	9	77.1	13.2
7	(B8) rejected skin graft from line 15	4	19.5	3.9	9	79.0	18.2
7	(B4) autograph surviving	3	14.5	1.6	8	133.2	24.5
7	(B19) not operated	5	11.4	1.2	4	55.2	16.6
15	(Y9) rejected skin graft from line 7	12	19.2	2.2	3	47.3	—
15	(Y2) skin graft from line 7	10	36.1	6.9	2	13.1	—
15	(Y24) not treated	9	27.0	4.8	3	17.3	—

donor (AKR) was previously immunized with the hybrid cells. The failure to obtain similar results in the chicken may be due to an insufficient amount of homogeneity in the two lines used. In preliminary studies, 50% of skin grafts performed at 10 days post-hatching persisted for at least 5 months in line 7 hosts and for at least one to two months in line 15 hosts. Studies of the effects of spleens from chickens which have rejected a number of skin grafts from several different donors from the opposite line are in progress.

DISCUSSION

The extensive investigations of many laboratories, including our own, have led to the conclusion that the embryonic splenomegaly induced by grafts or injections of homologous spleen cells involves at least two major steps: donor cells actively pervade the host's reticulo-endothelial tissues, there to proliferate and mount an immunologic reaction against the host. The pattern of this attack in the host's chorioallantoic membrane and spleen has been followed by DeLanney and Ebert (1959a, 1959b; see also DeLanney, Ebert, Coffman and Mun, 1962). In spleens of hosts receiving grafts of adult chicken spleen, a pronounced shift toward granulopoiesis is observed by the eleventh day, followed by accumulation of mucopolysaccharide, breakdown of the vascular bed, and necrotic and fibrotic foci.

Biggs and Payne (1961) observed similar pathologic changes in the host spleen following inoculation of chick embryos with competent adult cells: extensive proliferation of reticulum cell foci, and the formation of blast cells and granulocytes. This phase is followed by a lymphoid transformation of the reticulum cell foci.

These observations and others suggest that although some of the cells initially transferred from the donor graft to the host spleen proliferate, cells of the host proliferate also. Biggs and Payne observed that mitotic figures of both donor and host origin were present approximately in the proportion of 1:1 in spleens enlarged five- to twenty-fold. From cytological studies of changes in the host's spleen following inoculation of adult chicken blood, they argue that the reticulum cell foci, together with some of the blast cells, are of donor origin and that the majority of blast cells and developing granulocytes are of host origin, a conclusion in good agreement with the observations of the authors (Ebert, 1959; DeLanney, Ebert, Coffman and Mun, 1962). Although his earlier writings emphasized the proliferation of donor cells, Burnet (Burnet and Burnet, 1961; Warner and Burnet, 1961) now agrees that much of the proliferation is of cells of the host.

Most of the observations presented in the foregoing pages bear directly on the first phase of the reaction, lending support to the general argument advanced for its immunologic nature. For these, further discussion would be redundant. However, a few of the findings depart sufficiently from the expected to open new questions for discussion.

Earlier, one of us (Ebert, 1961b) had argued that the serial transfer experiments, using embryos of non-inbred lines, supported the idea that not only was the embryonic environment capable of supporting immune reactions, but also that a line of cells derived from the very first graft generation matured immunologically in that environment. Although the experiments reported herein with inbred lines do not require a major change in that view, it is necessary to state that it is not possible to argue for the derivation of the effective cells from the initial graft. What appears to be necessary is the accumulation of a threshold number of homologous cells; under the grafting conditions employed, one transfer is insufficient (*cf.* Howard and Michie, 1962).

Although we recognize that tolerance can be induced in adult animals (Rubin, 1959; Shapiro, Martinez, Smith and Good, 1961), it seems unlikely that the anomalous reduction in effectiveness of spleen taken from pre-immunized animals could be a degree of unresponsiveness as a consequence of competent cells introduced into an excess of antigen. Possibly, as an alternative explanation, the concept of allergic death (Boyse, 1959; Gorer and Boyse, 1959) may be advanced. Pre-immunized cells, exposed to antigen, if not immediately after implantation, at least upon invading the host tissues, undergo hyperactivity, resulting in their death. A test of this idea would be the examination of spleens of host embryos at time intervals after grafting shorter than the usual 5 days; if this argument were correct, one would expect a burst of donor cell proliferation, with early death of these lines.

Finally, we may comment briefly on the nature of the host's reaction. It is necessary to revive one of the several explanations which Billingham (1959) described as "ingenious" (p. 951). We do not believe that the development of the graft-versus-host concept has provided the "final solution" to the problem of homologous splenomegaly. The emphasis on donor cell proliferation (Billingham, 1959; Burnet and Burnet, 1960; Simonsen, 1957) resulted in a lack of interest in the host's response (Ebert, 1951, *et seq.,* reviewed 1959b; DeLanney, Ebert, Coffman and Mun, 1962). Earlier we advanced preliminary findings which suggested an incomplete immune reaction on the part of the host (Ebert and DeLanney, 1960; DeLanney, Ebert, Coffman and Mun, 1962). Although we have no reason to doubt that argument, it has become increasingly clear that it is not a *sufficient* explanation.

Undoubtedly, the sum total of evidence requires that the first step of the reaction be an immune graft-versus-host reaction. This results in the initiation of the second step, an intense proliferation of host cells due to the release of growth-promoting substances from the immunologically damaged host cells. That damage, irrespective of the mechanism by which it is produced, can lead to growth promotion is now a well-established fact (Abercrombie, 1957; Argyris and Argyris, 1959, 1962; Bullough and Laurence, 1960).

Concomitantly with host cell hyperplasia the donor cells also continue to proliferate due to the host antigenic stimulus. With increase in the number of donor cells, a more intense immune attack on the host occurs, leading in turn to further damage, and to further proliferation of host cells. It is apparent that these two interactions will result in massive growth of the spleen. The relative contribution of host and donor cells to splenomegaly will vary, and we would expect a greater contribution from host cells since they are present in much larger numbers. Thus the wide variations in the relative contributions of host and donor cells to splenomegaly experimentally observed become understandable, and, in fact, expected.

This hypothesis helps us to understand another feature of splenomegaly which so far has remained unexplained, that of fibrosis and its associated metachromasia (Ebert and DeLanney, 1960). Connective tissue proliferation is to be expected after damage of an organ, along with parenchymal proliferation, since connective tissue is stimulated by damage just as parenchymal tissue is (Abercrombie, 1957). In addition, such connective tissue proliferation is usually associated with increases in mucopolysaccharides which are responsible for the intense metachromasia (Washburn, 1960). We do not know if the stimulation of connective tissue proliferation is due to relatively nonspecific growth-promoting substances released by damage (Abercrombie, 1957; Swann, 1958), or whether the graft directs a specific antibody attack on the connective tissue cells which in turn release tissue-specific growth-promoting substances. That growth-promoting substances released by damage might be tissue-specific is suggested by the recent work of Argyris and Argyris (1962), and Bullough and Laurence (1960).

The actual mechanism of growth promotion leading to splenomegaly is unknown, but it is related clearly to the mechanism advanced by Ebert (1951, 1954), which was in turn related to Weiss's template-antitemplate theory of growth regulation. According to this view (reviewed, Weiss, 1960), the introduction of disintegrating cells should release specific templates which would "combine with, or otherwise trap, homologous antitemplates, their presence in the pool will entail a temporary lowering of antitemplate concentration, hence again a spurt of growth in the homologous cell strains of the host" (p. 65). Or templates might be incorporated directly into homologous cells, accelerating the growth rate. Partial necrosis of an organ (which is precisely what is observed as a consequence of the graft-versus-host reaction) will have the same effect as partial removal, *i.e.,* compensatory growth. Hence the stimulating effects of tissue-specific ribonucleoprotein fractions (Ebert and DeLanney, 1960; DeLanney, Ebert, Coffman and Mun, 1962) and other lines of evidence (reviewed, Ebert and Wilt, 1960) must be re-examined in this light.

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SUMMARY

1. As demonstrated by their capacity to induce splenomegaly and by tritium-thymidine labeling, some of the cells of chorioallantoic grafts of adult chicken spleen colonized the chorioallantois, spleen, and other organs of the host embryo within two days.

2. The capacity of the embryonic environment not only to support immune reactions but also to permit maturation of mechanisms of immune response was demonstrated by the serial propagation of embryonic spleen cells in non-inbred embryos. A cumulative response was obtained, beginning with the fifth or sixth transfer, approximately paralleling the normal development in the chicken of the ability to elicit splenomegaly.

3. However, stimulation of the host spleen was not obtained by the serial propagation of embryonic spleen cells in inbred embryos nor in a series in which the single initial donor was derived from a different inbred line. This suggested that the accumulation of a threshold number of reactive cells is necessary for the stimulation.

4. Induction of mutual interbreed "tolerance," as indicated by reduced effectiveness of adult chicken spleen to induce splenomegaly, was not obtained by previous chorioallantoic grafts of embryonic spleen.

5. The pre-immunization of adult chickens of one inbred line by skin homografts from a second line did not render the former's spleen capable of an enhanced reaction but, instead, reduced its effectiveness to elicit host spleen enlargement. It was suggested that such hyperimmunized cells undergo allergic death.

6. Attention is redirected to the proliferation of cells of the host following an initial graft-versus-host reaction. It is again suggested that this granulocytic response is a tissue-specific growth reaction resulting from the liberation of cell products in necrotic foci created in the initial immune reaction.

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INFLUENCE OF HOSTS ON THE BEHAVIOR OF THE COMMENSAL CRAB PINNOTHERES MACULATUS SAY[1]

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Analysis of factors binding the complex host-commensal relationship is a prelude for an understanding of the community integration and interspecific interactions of marine animals. Extensive accounts of host-commensal partnership have been given by Caullery (1952), Davenport (1955) and Dales (1957). Davenport and his collaborators (1950, 1951, 1953a, 1953b, 1957, 1958 and 1960) showed that a variety of commensals were attracted to their hosts by some diffusible substance released by the host animals. Lucas (1947) pointed out that external metabolites, "ectocrines," play a significant role in establishing the commensal symbiotic relationships.

The commensal crabs, *Pinnotheres maculatus* Say, are present in bay scallops, *Aequipecten irradians concentricus* Say, and penshells, *Atrina rigida* Solander, in Alligator Harbor, Franklin County, Florida. Johnson (1952) demonstrated chemotaxis in pinnotherid crabs with the *Dissodactylus-Mellita* partnership. In view of the frequent occurrence of the crabs in bay scallops and penshells it was decided to examine the host-commensal partnership and to determine to what extent the host affect the crabs.

The authors thank Dr. S. K. Katti for suggesting the statistical method used in the study.

MATERIALS AND METHODS

Bay scallops and penshells were collected in Alligator Harbor, Franklin County, Florida, near the marine laboratory of the Oceanographic Institute, Florida State University. The host animals were maintained in the Oceanographic Institute laboratory in running sea water tables and examined for the presence of crabs immediately after being brought in. The crabs were removed from the hosts and kept in separate running sea water aquaria. In isolation, the crabs were as healthy and active after a period of time as those just removed from the hosts.

To study the attraction of crabs to the hosts, a circular choice apparatus was constructed with plastic material. The principle of circular choice apparatus is well described, with figures indicating the direction of water currents, by Bartel and Davenport (1956). The only difference in the circular choice apparatus used in the present study is that it is larger in size to suit the experimental animals under investigation.

Water circulation in the apparatus was maintained at a steady rate, determined by preliminary flow tests to eliminate the influence of turbulence on the behavior of

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TABLE I

Infestation of bay scallops by Pinnotheres maculatus *in different months of a year*

Month	Scallops examined	No. scallops infested with		% total infestation
		Males	Females	
October, 1957	32	1	7	25.0
June, 1958	15	1	2	20.0
July	142	16	51	47.1
August	98	11	29	40.8
September	7	0	2	28.6
October	18	4	3	38.8
November	13	1	2	23.1

crabs. The water in the apparatus was allowed to circulate for some time before the experimental animals were introduced. A host was introduced first into one of the radial chambers and the system was allowed to come to equilibrium. Then the crabs were introduced into the central chamber. (Hosts were introduced before the crabs because it was found that if both host and commensals were placed at the same time, the crabs began random choice before the responsible factor from the host had sufficient time to become equilibrated in the water circulation of the choice apparatus.) The experiments were conducted in subdued light since the crabs were found to be negatively phototactic; under brightly lighted conditions they remained motionless at the margins of the central chamber.

It was found that 12 hours was sufficient for crabs to make their choice and the number of crabs in each radial chamber was counted after this period. Before and after each experiment the apparatus was thoroughly cleaned with sea water. The behavior of the crabs while seeking hosts was noted. The temperature ranged between 28 and 31° C. during the experimental period. The results were analysed by χ^2 formula (Nass, 1959) to test the significance of distribution among the radial chambers.

TABLE II

Comparison of the average sizes of scallops infested and not infested with crabs

Date of collection	Infested		Not infested		Difference in size between infested and uninfested scallops, mm.
	No. scallops	Average size, mm.	No. scallops	Average size, mm.	
6/27/58	3	37.4	12	40.1	+2.7
7/7/58	5	44.9	18	42.9	−2.0
7/23/58	27	43.9	38	47.5	+3.6
7/30/58	26	49.8	20	52.0	+2.2
8/5/58	17	50.2	15	51.3	+1.1
8/13/58	5	56.2	20	54.7	−1.5
8/23/58	13	54.8	28	57.1	+2.3
9/14/58	2	62.3	5	58.3	−4.0
10/5/58	7	57.9	12	61.8	+3.9
11/14/58	3	66.1	9	64.2	−1.9

OBSERVATIONS

The commensal crabs live between the gill folds in the mantle chamber of scallops and penshells. Rathbun (1917) described the females of this species as commensals, whereas the young stages of males are free-living. The occurrence of male and female crabs in 1957–1958 collections of scallops is shown in Table I. Crabs of both sexes were most abundant during the summer months, and gravid females were found in the same period.

The feeding behavior of the crabs was similar in general to that described for other species of Pinnotheridae (Coupin, 1894; Orton, 1921; Stauber, 1954). Stauber (1945) observed that *Pinnotheres ostreum* caused gill erosions and thickening of the oyster host gills. Parts of the gills of scallops were broken off by the movements of the crabs within the mantle chamber. The average sizes of scallops infested and not infested with crabs are shown in Table II.

EXPERIMENTAL RESULTS

1. Distribution of crabs in choice apparatus in the absence of hosts

The crabs were placed in the central chamber of the choice apparatus without hosts in the radial chambers and the distribution at the end of the experimental period was noted. The crabs were sluggish and a few remained in the central chamber at the end of the experiments without making any choice. The χ^2 analysis of results (Table III) indicated that the crabs showed no preference for any of the chambers.

TABLE III

Distribution of commensal crabs, Pinnotheres maculatus, *in the radial chambers of the choice apparatus in the absence of host influence*

Expt. no.	Crabs		Distribution in radial chambers						χ^2	Critical χ^2 value for 5%
	Tested	Made choice	1	2	3	4	5	6		
1	13	9	4	0	1	1	0	3	9.37	11.98
2	15	13	0	2	2	2	1	6	9.83	11.67
3	14	10	1	5	1	0	1	2	9.55	11.98
4	16	16	3	2	5	2	1	3	3.33	11.52

2. Attraction of crabs to host scallops

When a scallop was present in one of the radial chambers, the crabs were very active and moved freely in the central chamber. At the end of the experiments, the crabs were not homogeneously distributed in the radial chambers (Table IV). The crabs showed a statistically significant ($P > .05$) preference for the chamber containing the host, although in one of the seven tests the distribution was random.

The crabs required considerably less time to make their choice than they did in the control experiments. They gathered around the host chamber one after another or in groups. Crabs moving towards the non-host chambers sometimes

TABLE IV

Attraction of commensal crabs to bay scallops

Expt. no.	Crabs		Distribution in radial chambers						Host vs. non-host	
	Tested	Made choice	1	2	3	4	5	6	χ^2	Critical χ^2 value for 5%
1	8	8	(5)	1	0	0	1	1	11.25	3.57
2	12	12	0	(9)	3	0	0	0	26.98	3.64
3	9	9	1	(7)	1	0	0	0	22.05	3.57
4	11	11	2	0	(3)	0	3	3	0.82	3.64
5	10	10	0	1	0	0	0	(9)	41.61	3.61
6	20	19	2	1	0	(11)	3	2	21.52	3.57
7	16	16	1	1	2	1	(8)	3	12.29	3.68

Parentheses indicate chamber containing bay scallop.

reversed their direction of movement and moved directly into the host chamber. When the crabs moved to an opening leading to a non-host chamber, they remained at the opening for a long time before they made their choice, sometimes moving away from the openings. After entering the host chamber, the crabs gathered around and under the scallops. Some climbed on the upper valve of the scallop and made attempts to enter the host mantle chamber. While some of the crabs gained immediate entry into the host, others were caught between the valves of the host when it contracted, gaining entry when the scallops later opened their valves.

3. *Response when the host water is siphoned into one of the radial chambers*

These experiments were designed to find out if the crabs would respond to water coming from an aquarium containing a host scallop. Water from an aquarium containing a host was siphoned into one of the radial chambers of the choice apparatus. The distance of the host from the crabs in the central chamber was approximately six times greater than in the previous experiments in which the host was placed in a radial chamber. The rest of the procedure was the same as described in the general methods.

Results shown in Table V indicate that the attraction is reduced as compared to

TABLE V

Choice of crabs when the host water is siphoned into one of the radial chambers

Expt. no.	Crabs		Distribution in radial chambers						Chamber with host water vs. non-host	
	Tested	Made choice	1	2	3	4	5	6	χ^2	Critical χ^2 value for 5%
1	16	14	3	2	(5)	1	1	2	3.47	3.64
2	14	14	(4)	2	2	1	4	1	1.36	3.64

Parentheses indicate chamber into which host water is siphoned.

TABLE VI

Response of crabs to scallop shells with attached animals

Expt. no.	Crabs		Distribution in radial chambers						Chamber with host shell vs. those without host shell	
	Tested	Made choice	1	2	3	4	5	6	χ^2	Critical χ^2 value for 5%
1	14	14	(5)	2	2	3	1	1	3.50	3.68
2	15	12	5	2	0	1	(4)	0	2.28	3.64
3	12	10	0	1	(7)	2	0	0	19.24	3.61
4	15	10	3	0	3	(3)	0	1	0.12	3.61

Parentheses indicate chamber containing the scallop shell.

that of the chambers containing live hosts. The Chi square test showed that crabs were distributed homogeneously in all six radial chambers; the distribution of crabs in the chambers was random.

4. *Response of crabs to host shell with attached animals*

A variety of sessile animals attach to the outside of the shell of scallops. Scallop shells with attached animals were washed with sea water after the soft parts of the scallops were removed and were tested to find whether the crabs would be attracted to them. The results (Table VI) show that there is no significant attraction of crabs to the shell with attached organisms, except in one of the experiments there is a significant attraction of crabs. This could have resulted from insufficient washing of the shell after removing the soft parts.

5. *Response of male commensal crabs to host scallops*

Adult males are commensals in their relation with the host, whereas the early male stages are free-living. Experiments were planned to investigate whether the males, removed from the host scallops, respond to the host in the same manner as the females. The results are summarized in Table VII. The males were very active in the presence of the host and occasionally swam within the central chamber of the choice apparatus.

TABLE VII

Response of male commensal crabs to bay scallops

Expt. no.	Crabs		Distribution in radial chambers						Host vs. non-host chambers	
	Tested	Made choice	1	2	3	4	5	6	χ^2	Critical χ^2 value for 5%
1	21	21	3	0	0	0	2	(16)	52.18	3.72
2	17	14	3	(8)	0	2	0	1	15.55	3.64
3	13	13	1	1	2	(9)	0	0	24.41	3.64
4	13	13	2	2	0	1	(8)	0	17.89	3.64

Parentheses indicate chamber with host.

TABLE VIII

Attraction to penshells of crabs removed from bay scallops

Expt. no.	Crabs		Distribution in radial chambers						Host chamber vs. non-host chambers	
	Tested	Made choice	1	2	3	4	5	6	χ^2	Critical χ^2 value for 5%
1	16	16	0	6	(10)	0	0	0	23.23	3.68
2	15	15	0	3	0	0	(11)	1	33.29	3.68
3	16	16	1	0	(11)	1	1	2	30.87	3.68

Parentheses indicate chamber containing penshell.

Chi square analysis of the results indicates that the males were attracted to the scallops. Since no female crabs were present, the attraction of the male crabs appears to be entirely to the host.

6. *Attraction to penshells of crabs removed from scallops*

These experiments were performed to determine whether crabs originally removed from scallops would be attracted to a second host, *Atrina rigida.* Crabs living in the scallops and the penshells are morphologically similar. Penshells of approximately the same weight as scallops used in the earlier experiments were placed in the radial chamber of the choice apparatus and the distribution of the crabs at the end of the experiments was noted. The results (Table VIII) indicate that the crabs were strongly attracted to the penshells.

7. *Preference of crabs between the two hosts, scallops and penshells*

The two hosts, penshells and scallops, were placed in two non-adjacent radial chambers of the choice apparatus and the crabs were introduced in the central chamber. Crabs obtained from scallops showed no statistically significant preference for penshells than for the scallops (Table IX).

TABLE IX

Response of crabs, removed from bay scallops, to penshells and bay scallops when both are present in two separate chambers of the choice apparatus

Expt. no.	Crabs		Distribution in radial chambers						Hosts vs. non-host chambers		Penshells vs. bay scallops	
	Tested	Made choice	1	2	3	4	5	6	χ^2	Critical χ^2 value for 5%	χ^2	Critical χ^2 value for 5%
1	15	15	1	1	(8)	0	3*	2	14.49	5.81	2.50	3.42
2	15	15	(6)	2	1	5*	1	0	10.58	5.81	0.09	3.42

Parentheses indicate chamber with penshell; * indicates chamber with bay scallop.

DISCUSSION

The attraction of commensals to their hosts in response to some diffusible substance or substances released from hosts was demonstrated by Welsh (1930), Thorpe and Jones (1937) and Davenport (1950, 1953a). The present experiments showed that the commensal crab, *Pinnotheres maculatus,* is capable of recognizing its hosts, *Aequipecten irradians* and *Atrina rigida,* under the described experimental conditions. The active movements of the commensal crabs in the presence of the hosts seem to be stimulated by some attractant from the host. The attraction of commensals to the host scallops decreased when the hosts were not directly introduced in the radial chamber of the choice apparatus. This suggests that perhaps a spatial proximity of hosts to commensals is necessary for demonstration of attraction under experimental conditions. The decreased attraction could have resulted either from a gradient or a highly diffusible nature of the attractant.

The absence of attraction of commensals to empty host shells, with attached epizooites, indicates that the source of the attractant is the soft parts of the scallops.

The experiments with males of *P. maculatus* demonstrated conclusively that their response to scallops is equal to that of the females of the same species. It is not known from the present study how the free-living early stages of males change to commensal habit in their adult stage. Experiments with free-living early-stage males might reveal the nature of this change.

Crabs removed from scallops were attracted readily to *Atrina rigida,* another host which inhabits the same general locality as the scallops. The results of experiments indicate that both scallops and penshells release attractants that stimulate the crab to seek the hosts. The attraction of crabs from scallops to both the hosts appears to be equal when both are simultaneously tested for response. Crabs living in the scallops and those in the penshells are morphologically similar, and crabs from scallops are not physiologically host-specific. Reciprocal experiments with crabs obtained from penshells should elucidate the specificity of these commensal crabs.

SUMMARY

1. Experiments using a circular choice apparatus showed a statistically significant attraction of commensal crabs, *Pinnotheres maculatus,* to bay scallops, *Aequipecten irradians concentricus,* and penshells, *Atrina rigida.*

2. The adult males of *P. maculatus* removed from bay scallops showed a significant attraction to the host.

3. When tested for preference between the two hosts, crabs removed from bay scallops showed no preference for one host over the other. The attraction of crabs to both the hosts was statistically significant. Experiments suggested that the crabs removed from scallops are not host-specific.

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REPRODUCTION OF THE POLYCHAETE GLYCERA DIBRANCHIATA AT SOLOMONS, MARYLAND[1]

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Remarkably little is known about the life history and the ecology of the errant polychaete family Glyceridae. Basic information of this nature is needed not only to allow a synthesis of such isolated data as are presently available, but also to provide the background necessary for experimental investigations. In view of this, the work of Klawe and Dickie (1957) on *Glycera dibranchiata* Ehlers in the Maritime Provinces is of particular interest, since it appears to be the only publication dealing with the biology of a glycerid worm. Commonly known as the "bloodworm" or "beak-thrower," this species is a favorite bait of salt-water sport fishermen, whose demand has made it of some commercial importance in Maine and the Maritime Provinces. The studies of Klawe and Dickie were undertaken to obtain information relating to questions of bloodworm conservation, and their report contains many original observations. But the chief value of their contribution lies, perhaps, not so much in its extensive data as in its indication of the numerous problems still requiring considerable study.

Of particular importance are the gaps persisting in our knowledge of glycerid reproduction, many aspects of which have remained largely a matter of conjecture. It is with these deficiencies in the case of *Glycera dibranchiata* that the present study is concerned. Intended to enlarge upon the work of Klawe and Dickie, this report deals with the breeding season, swarming and epitoky of *G. dibranchiata* in more southern waters, and uses histological findings to supplement field observations. Gametogenesis and early development will be considered in a separate paper.

METHODS

Most of the information presented here is based on work conducted at the Chesapeake Biological Laboratory at Solomons, Maryland, from the last week of June, 1960, to the early part of February, 1961. Unless otherwise stated, all specimens were collected from the waters immediately surrounding Solomons Island, situated in the mouth of the Patuxent River, about two miles from its entrance into Chesapeake Bay (Fig. 1). Some of the hydrographic features of this area have been described by Nash (1947) and Beaven (1960). Since the

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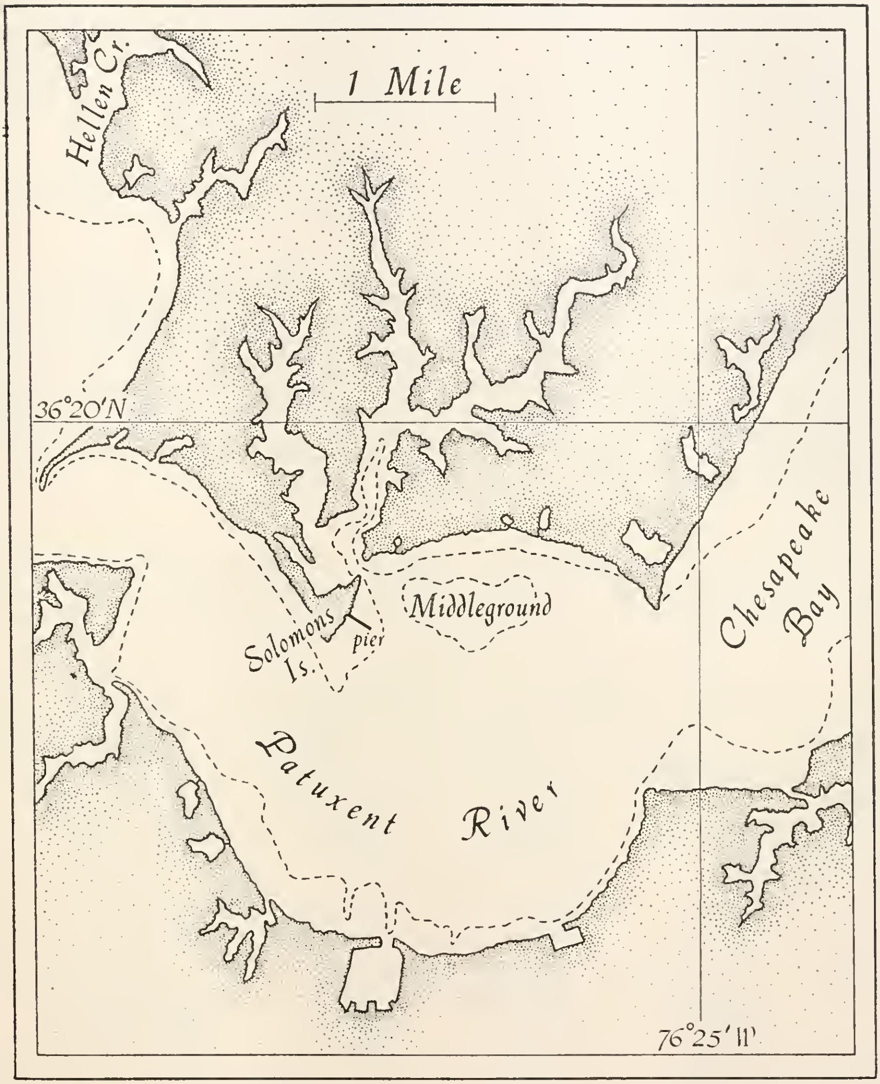
1 Mile
Hellen Cr.
36°20′N
Solomons Is.
pier
Middleground
Chesapeake Bay
Patuxent River
76°25′W

Figure 1. Location of Solomons Island and other areas mentioned in the text. Broken line indicates the 12-foot bottom contour.

mean tidal amplitude at Solomons is only 1.2 feet, there is practically no intertidal zone; all collecting, therefore, was done with a Maryland soft clam dredge (see Manning, 1959), operated in 6–10 feet of water. This method, using a wire mesh conveyor belt to bring specimens up from the bottom, proved satisfactory for obtaining 2–4 dozen uninjured worms in a relatively short period of time.

Living worms narcotized in magnesium chloride were measured by the method of Klawe and Dickie, who used a watertight trough, V-shaped in cross-section, with a ruler attached to one side. Some measurements were obtained from fixed material and will be so indicated. Narcotized animals were fixed in Bouin's fluid and stored in a preservative consisting of 2 parts ethyl alcohol, 1 part distilled water and 1 part glycerine. For general examination, paraffin sections 4–7 μ thick were stained in Ehrlich's hematoxylin and eosin.

Other methods used in connection with specific problems will be described in the appropriate sections.

BREEDING SEASON

Table I summarizes the available information on reproductive periods in several species of *Glycera*. For *G. dibranchiata,* Klawe and Dickie have indicated mid-May as the probable time of maximum reproduction in Nova Scotia and Maine. The present study was begun near the end of June, too late to determine the presence or absence of a spring spawning. Other records, however, suggested the possibility of a second breeding season for this species at Solomons, and an autumn spawning did in fact occur.

Previous reports for Solomons

Three earlier reports of breeding activity for *Glycera* were found in records at the Chesapeake Biological Laboratory. One of these is a brief anonymous note

TABLE I

Breeding seasons of Glycera *species*

Species	Locality	Time of year	Evidence	Source
G. alba	The Sound	Late autumn?	Larvae in winter	Thorson, 1946
G. americana	Woods Hole Maryland Solomons	Summer Spring–Summer December	Adults at surface Swarming Swarming	Pettibone (in press) Anonymous, 1948 Beaven (see text)
G. capitata	Norway	May–July	Epitokes at surface	Støp-Bowitz, 1941
G. convoluta	Naples Algiers Plymouth	May April June–August	Spawning Epitoke at surface Ripe gametes	Lo Bianco, 1909 Gravier and Dantan, 1928 Fuchs, 1911
G. dibranchiata	Woods Hole Maine and Canada Maryland Solomons Solomons Solomons	August; January April–June Spring–Summer July November December	Adults at surface Ripe gametes Swarming Swarming Swarming Swarming epitokes	Pettibone (in press) Klawe and Dickie, 1957 Anonymous, 1948 Myers (see text) Beaven (see text) This paper
G. lapidum	Algiers Concarneau Norway and Sweden	February December–April August?	Epitoke at surface Epitokes at surface Pre-epitokes in July Young in September	Gravier and Dantan, 1928 Fage and Legendre, 1927 Støp-Bowitz, 1941 Arwidsson, 1898
G. nana	British Columbia	Autumn	Swarming	Berkeley and Berkeley, 1948
G. robusta	Monterey Bay	Spring–Summer	Ripe gametes	MacGinitie, 1935
G. rouxii	Concarneau Banyuls Norway and Sweden	October August October?	Epitoke at surface Epitoke at surface Pre-epitokes in September	Fage and Legendre, 1927 Fage and Legendre, 1927 Støp-Bowitz, 1941
G. siphonostoma	Naples	December–April	Mature adults	Lo Bianco, 1909
G. ?sphyrabrancha	Puerto Rico	October	Ripe adults at surface	Allen, 1957
G. tesselata	Algiers	October–November	Epitokes at surface	Gravier and Dantan, 1928

appearing in a 1948 issue of the Maryland Tidewater News and describing swarming of these worms "in the tidal waters of Maryland" during late spring and early summer of each year. It also states (p. 4) that "there are two species of *Glycera* found along our coast, both of which carry on the curious antics reported above." Presumably the species referred to would be *G. dibranchiata* and *G. americana*. The other two reports of swarming were found in a card file of invertebrates occurring near Solomons. One gives the following information: "*Glycera dibranchiata;* large numbers swimming off CBL pier, presumably this species; one specimen identified; July 15 [no year]; Marvin Myers." The other entry reports *G. dibranchiata* swarming in the same area on the nights of December 4–7, 1944; apparently *G. americana* was also present the first of these nights. In none of these cases has it been possible to verify the identity of the worms. The December, 1944, swarms, however, were witnessed by Mr. Francis Beaven, currently a member of the Laboratory staff, who was able to provide some further details concerning the event. There seems little reason to question that in this case, at least, the generic identification was correct. If the benefit of the doubt is extended to the other two reports, it must be concluded that *Glycera* has two breeding periods a year in this locality.

Rate of sexual development

Between late June, 1960, and swarming time in early November, 75% of the worms examined histologically had gonads in various stages of development. These specimens, 55 in all, represent ten samples taken the following numbers of days before the first swarm: 129, 90, 77, 70, 58, 45, 36, 16, 8 and 4. Relaxed length of the worms ranged from 7 to 26 cm., with the majority falling into the 16–20 cm. group. Although these limited samples allow only tentative generalization, two observations should be mentioned. First, there seemed to be no correlation between specimen length and presence of gonads, though the gonads of shorter worms were generally smaller. Secondly, contrary to expectation, the frequency of mature specimens in each sample diminished as swarming time drew closer. This could, perhaps, indicate a migration of mature worms away from the usual collecting area into shallower water, where swarming appeared to be more concentrated. But both of these observations need to be checked by statistical treatment of larger samples.

The earliest definite sign of approaching maturity occurred in mid-September, 45 days before swarming. Two of the specimens fixed then contained sperm plates and a third contained eggs in the coelom. The appearance and the small number of these free gametes suggested that their release from the gonads had just recently begun. This agrees reasonably well with the observations of Klawe and Dickie, who found immature eggs free in the coelom of some worms in late August. Evidence of a more advanced degree of maturity appeared about one month later, in a specimen fixed 16 days before swarming. This was a female with eggs almost completely filling the body cavity and with no remaining gonad tissue. The atrophy of the gut and body wall that accompanies sexual maturity had already begun but was not yet pronounced. The sample collected four days before swarming included worms in final stages of maturation. Two females and one male shed gametes when handled in the laboratory. Although the sperm plates released by the male did not break up into individual sperm, some of the gametes must have been ripe, since an attempt at fertilization gave a small number of cleaving eggs that developed into swimming blastulae.

If biannual reproduction is assumed, these observations may be tentatively interpreted as follows: Of the worms collected in summer, those with well developed gonads probably represented the fall breeders of 1960; those with poorly developed gonads, the spring or summer breeders of 1961; and those with no gonads, the fall breeders of 1961. It would then follow that complete sexual development requires about one year. The observations also indicate that young gametes of both sexes are released from the gonads into the coelom at approximately the same time and ripen within the following 6 to 8 weeks.

Length of breeding season

It is difficult at present to suggest the limits of the reproductive seasons at Solomons. Both the anonymous article and Myers' notation indicate that the earlier period may center around June and July. The fact that no mature worms were found at the beginning of the present study (the end of June) is not incompatible with this suggestion. If actual spawning is of short duration, a matter of a few days to a week, then it could have taken place in its entirety during the early part of June. Furthermore, it has already been mentioned that mature worms became scarcer in samples dredged nearer the time of swarming; thus it is possible that the number of mature worms had similarly decreased in the collecting area during late June. Any swarming activity that might have occurred in June or July could easily have been overlooked. The information available for the autumn breeding season is more definite, though still insufficient to form any hard and fast conclusions. In both cases, namely December, 1944, and November, 1960, observed swarming was limited to four successive days. Whether this coincidence is purely fortuitous, or whether it reflects a high degree of reproductive synchrony cannot at present be settled. Nor is there any way to determine that these were the only spawning events during the fall months of 1944 and 1960.

Environmental factors

Temperature conditions of surface water at Solomons for a 20-year period have been summarized by Beaven (1960). These records show mean temperatures ranging from 3.3° C. in February to 26.7° C. in August, with extremes of −0.8 and 31° C. The greatest difference between surface and bottom temperature occurs in the spring, when readings may be about three degrees lower at 17 feet (Nash, 1947), and eight degrees lower at 60 feet (Beaven, 1960). During the fall months water is generally slightly warmer at the bottom than at the surface. There appears to be little correlation between absolute temperature and the suggested breeding periods, except that temperature is approaching its maximum during June and July, and its minimum during November and December. In the two-week period before swarming, water temperature fell gradually from 10.3 to 5.5° C. in 1944, and from 18.7 to 13.8° C. in 1960. The average daily difference was −0.36° C. in both years, but this is of questionable significance. Otherwise the data suggest only that final sexual maturity can be attained within a fairly wide temperature range.

Two attempts were made at Solomons to determine whether temperature change can affect gonad development in *Glycera*. The first of these took place in August, when ten worms were kept at a temperature below the average 26° C. of the water pumped into the laboratory. The arrangement for maintaining a flow of fresh water at a constant low temperature proved unstable, allowing irregular variations within a range of 12° to 20° C. After about three weeks seven of the worms had died, but all three survivors had gonads approximately twice the size of those in freshly collected worms fixed at the same time. The second attempt to observe effect of temperature change was made in January, under somewhat better conditions. Six animals were kept in separate containers placed in a cold-water bath maintained at 14° C. by a thermostatically controlled heating coil. No attempt was made in this case to provide running water to the test animals; rather, the water in the containers was changed daily. A second group of worms, serving as a rough control, was kept at about 4° C. in an aquarium supplied with running water from the pump system. After two weeks both groups were fixed. Of the six worms exposed to the higher temperature, four were in advanced stages of maturity. In these, the coelom contained well developed eggs or sperm plates, there was little or no remaining gonad tissue, and atrophy of the gut and body wall was pronounced, in two cases exceeding by far the degree of atrophy found in any of the swarming specimens examined. None of the animals kept at 4° C. showed free gametes or gonads markedly larger than those found in freshly collected worms. Although inconclusive, these results strongly suggest the importance of temperature in regulating the rate of sexual maturation in *Glycera*.

Temperature is generally regarded as a critical factor in determining the reproductive period of many marine invertebrates. Indeed, Orton (1920) concludes that other environmental conditions are of little significance. There is, however, a growing body of experimental evidence indicating that while temperature changes may accelerate gametogenesis and induce spawning, these responses depend to some degree on the physiological condition of the organism (*e.g.* Galtsoff, 1940; Loosanoff and Davis, 1952; Turner and Hanks, 1960). Furthermore, Thorson (1946) has pointed out that seasonal phytoplankton maxima cannot be excluded from the possible factors regulating reproductive activity in benthic invertebrates. Large plankton blooms in April and May, and smaller ones in September and October, have been reported for the waters around Solomons (Nash, 1947). These seasonal fluctuations occur shortly before the suggested breeding periods and hence, by augmenting the food supply, may well exert an indirect influence on the timing of reproduction in *Glycera*.

Breeding season in Canada and Maine

Klawe and Dickie conclude that bloodworms in Nova Scotia and Maine spawn only once a year, in the spring. But although they state that no observations were made in winter, it is not clear how long their observations were continued in autumn. It cannot be assumed that because two allopatric populations belong to the same species, their reproductive periods will coincide. The spawning of some nereids, for example, occurs earlier in southern than in northern latitudes, and even in the same locality, intertidal populations and those below mean sea level may spawn at different seasons (Herpin, 1926, 1928; Fage and Legendre, 1927). In addition to affecting the time of reproduction, the habitat apparently can in some polychaetes also influence the method of reproduction (Thorson, 1950). Thus it is entirely possible that bloodworms in Canada and Maine spawn but once, whereas those further south spawn twice a year.

Nonetheless there is reason to suspect that spawning may be a biannual phenomenon for the more northern members of *G. dibranchiata* as well. Two points reported by Klawe and Dickie tend to support this suggestion. First is their observation of free oocytes in the coelom of worms examined in late August, which approximates the present findings. Their report implies that these gametes continue development through the winter and are not shed until the following spring. Although there is evidence that some invertebrates can often store ripe gametes for long periods before releasing them (Herpin, 1928; Thorson, 1946), it seems equally possible that in Nova Scotia, as at Solomons, these germ cells complete growth within a month or so and are spawned in autumn. Secondly, Klawe and Dickie mention that swarming worms, caught in a herring fisherman's net off Nova Scotia in October, 1955, were identified as bloodworms by local worm diggers, who communicated this information to the authors. But Klawe and Dickie were unable to verify this report, and since collections made in the same locality the following September yielded only swarming nereids, conclude that the diggers' identification must have been erroneous. In view of the observations from Solomons, this conclusion should perhaps be reconsidered. It may finally be mentioned that specimens originating in Maine and obtained from bait stores at Washington, D. C., in late September and early October have often contained eggs resembling in size and appearance those spawned by ripe females at Solomons.

Although final resolution of this question will depend upon confirmatory observations of spring spawning at Solomons and fall spawning in the Maritime Provinces, the present evidence permits the suggestion that *G. dibranchiata* along the Atlantic coast reproduces twice a year. Should this be correct, it will be necessary to revise the conclusions on growth rate and life span arrived at by Klawe and Dickie. These authors find that their size-frequency curves show four distinct modes, which they interpret as successive age groups. Thus the mode centering around 5 cm. represents yearling worms, that around 16 cm. represents two-year-olds, and so on, to a maximum of about 31 cm. for four-year-olds. The sudden decline in frequency of worms three years old, in comparison with the frequency of two-year-olds, is taken as an indication that most bloodworms spawn and die as they reach their third year. But as Klawe and Dickie point out, these conclusions are based on the assumption that spawning occurs only once each year. If it is in fact a biannual affair, then the modes of the size-frequency curves would represent two year-classes rather than the four proposed by Klawe and Dickie, and their consequent deductions would have to be modified accordingly.

SWARMING

Several species of *Glycera* are reported to take up a brief pelagic existence at the time of spawning (see Table I). Although Klawe and Dickie found no evidence for such behavior in *G. dibranchiata,* they suggest that bloodworms may have a short nocturnal swarming period, as do many other errant polychaetes. In an effort to check this possibility, night observations using a 150-watt bulb suspended 18–20 inches above the water were conducted from the end of the Laboratory pier at

Solomons, about 700 feet from shore, over water 8–9 feet deep. This location was chosen primarily for its convenience, but also because dredging had indicated a good concentration of bloodworms in the vicinity. More than 40 such observations were made between June and November. Most of the first 20 fell within the last half of the lunar cycle in July, August and September; the others, in October and November, included all four lunar periods. A single night's session lasted two to three hours, usually between sunset and midnight, although several observations in October and early November were conducted at times between midnight and dawn. No bloodworms appeared at the surface during any of these periods, and the observations were discontinued after the first week of November.

Dates and areas of observed swarms

Swarming of *Glycera dibranchiata* was first noticed during the afternoon of 5 November 1960 by Mr. Hayes T. Pfitzenmeyer, a member of the Laboratory staff, who brought it to the author's attention. It was witnessed again on the two following afternoons, and although no personal observations were made on November 8, reports of other staff members indicated that swarming occurred on that day also.

On November 5, swarming took place over the Middleground (Fig. 1), a shoal area approximately 500 yards east of the pier, in water 1–3 feet deep. The extent of the swarm could best be gauged by the activity of gulls, large numbers of which congregated over this area and the north shore, diving toward the water and rising with worms dangling from their beaks. This swarm lasted from about 3:00 to 5:30 P.M. and was investigated from a small boat. One of the Laboratory staff members later mentioned seeing worms on the surface at approximately 4:30 this same day while trolling in 6–10 feet of water in the vicinity of Hellen Creek; he also remarked that the stomachs of striped bass caught by him were filled with worms. Swarming on November 6 began at 4:10 P.M., in shallow water around the pier, and was again investigated by boat. Concentrations of gulls were also noticed along the north shore and to a lesser extent over the Middleground. On November 7 swarming was indicated by gulls working in the same areas as the preceding day; the boat was not used this day, and observations were limited to activity around the pier.

At the time of swarming on these four days, water temperature ranged from 12.2 to 13.8° C., and the average salinity was 14.5–14.8‰. Weather conditions were generally agreeable, except for the first day, which was overcast and rainy.

Composition and density of swarms

Ten specimens, 14 to 20 cm. long (fixed), were collected during the swarms of November 5 and 6. Only one of these was a female, but the actual sex ratio is probably less disparate. In large samples of mature worms, Klawe and Dickie found a size range of 13 to 36 cm., with males outnumbering females by only 1.3 to 1. Gravier and Dantan (1928) report lengths of 5 to 18 mm. for swarming *Glycera tesselata* and state that males were very predominant.

Although swarming activity at Solomons extended over considerable areas, individual worms were remarkably dispersed, occurring approximately 3–5 yards apart, and technically it may be questioned that the term "swarming" applies in such a case. Whether or not this dispersion is typical of spawning bloodworms, however, remains to be seen. Apparently, pelagic breeders of other *Glycera* species have seldom been encountered in great numbers. Gravier and Dantan (1928) report collecting 25 and 48 specimens of *G. tesselata* on two different occasions, but this departs from the usual catch of one or two individuals recorded by other authors and by Gravier and Dantan for other species. The studies of Gravier and Dantan at Algiers resemble those of Fage and Legendre (1927) at Concarneau, and since in both cases observations were made shortly after sunset, these authors suggest that swarming maxima probably occurred later at night. Yet if swarming individuals of other species were as scattered as *G. dibranchiata* at Solomons, then it seems entirely possible that observations from a fixed point, such as the anchored boats used in the Algiers and Concarneau studies, would yield very few specimens, even during the height of swarming activity.

An increased density could be expected if swarming glycerids were positively phototropic and concentrated around the lamps used in nocturnal investigations. But there is little evidence indicating that *Glycera* shows a positive response to light. Although primitive epidermal photoreceptor cells have been described for some species (Stolte, 1932), members of this genus do not possess eyes, nor is there any sign that such organs develop at maturity. During some observations made at Woods Hole late in the summer of 1959, several males of *G. americana* were collected from the surface at night. These animals did not seem attracted to the light, but had apparently been carried in by the current. Experimental data are needed, however, to establish the nature of photosensitive responses in both mature and immature specimens of this group.

Swimming and shedding behavior

Swarming worms moved slowly, either at or just below the surface. Their method of swimming was completely different from that of immature animals, which advance through the water by executing a series of intricate vertical figures-of-eight, with the tail always leading the way. In contrast, swarming individuals swam head first, propelled by lateral undulations of the body. These movements resemble the type of swimming shown by *Nereis* and *Nephtys,* and it is likely that as in these genera, locomotory waves originate at the posterior end and pass forward along the body (Gray, 1939; Clark and Clark, 1960). In all observed instances the proboscis was retracted. There was no indication of any particular swimming pattern, such as the circling dances of some nereids.

Many of the worms seen at close range were shedding gametes in a steady white flow from the posterior end. Of the ten swarmers collected, two lack tail segments, and a third shows a tear in the body wall near the tail. In the intact individuals, there is a very small rupture on the dorsal surface just anterior to the pygidium. Evidently this is the avenue for gametal discharge in the majority of cases. At no time, either during swarming or in the laboratory, were gametes observed to issue from a rupture in the proboscis or anterior two-thirds of the body. However, Klawe and Dickie found that shedding in the laboratory, whether spontaneous or induced by a weak electric current, occurred with about equal frequency through the tail, the body wall or the proboscis. Judging from observation of animals spawning in the laboratory, it seems probable that a single individual releases all its gametes at one time. In a few cases, swimming activity of animals shedding in fingerbowls was interrupted by one or two short periods of quiescence during which gametal discharge ceased. Apparently, the elimination of gametes does not require the presence of a worm of opposite sex and may well be a mechanical process resulting from the muscular pressures exerted on the coelomic fluid during swimming. This in turn would bring about a decrease in turgidity, with correspondingly weaker swimming movements until the spent animal finally sinks.

The only previous account of shedding in *Glycera* observed under natural conditions in Allen's (1957) report that pelagic breeders of *G. ?sphyrabrancha* released their gametes in two streams, apparently from pores in the midbody region. Genital ducts are not known for this genus, and gametal discharge in other species is generally presumed to occur through an oral opening left by the dissolution of the proboscis. There is no record, however, that this has ever been witnessed during actual swarming. The assumption is based primarily on the occurrence of epitokes in which the proboscis is either extremely degenerated or totally absent. With few exceptions, this condition has been found in *G. capitata, G. lapidum, G. alba, G. rouxii* and *G. tesselata* (Arwidsson, 1898; Fage and Legendre, 1927; Gravier and Dantan, 1928; Støp-Bowitz, 1941). Fragments of mature glycerids have also been collected at the surface (Fage and Legendre, 1927; Gravier and Dantan, 1928; Allen, 1957). The indications are, therefore, that shedding in *Glycera* is by dehiscence. The area of rupture probably depends on the degree of muscular atrophy, which might vary in different species.

Environmental factors

At present it is impossible to indicate the environmental agents that induce swarming in mature bloodworms or to predict the circumstances under which such behavior could be expected. Information on temperature, salinity, weather and tidal conditions was compiled from records maintained at the Chesapeake Biological Laboratory. A comparison of these data for the reported December, 1944, swarms and those observed in November, 1960, revealed no remarkable similarities. The four swarming days in both years fell between full moon and last quarter, but the significance of this cannot be established on the basis of only two reports. Swarming in 1960 began somewhat later each day, nearly coinciding with maximum high water of the second tide, and thus suggesting that tidal influences may be at least partially responsible for the daily timing of reproductive activity. Although exact hours are not available, it is known that swarming on December 4–7, 1944, took place at night (Beaven; personal communication), and hence could have been similarly associated with the second daily tide, which on those dates reached high water after dark.

A relationship between tidal conditions and onset of spawning has been reported for some other polychaetes, and in a few instances may perhaps be involved in daytime swarming (Herpin, 1926, 1928; Korringa, 1947). The regulation of gametal discharge to coincide with rising water is not difficult to understand in the case of intertidal populations. But there seems to be no ready explanation for such timing in animals not exposed at low water, especially when the tidal amplitude is small, as at Solomons. It is unlikely, however, that tidal movements alone could be responsible for stimulating swarming in *Glycera;* such behavior is more probably dependent upon the interaction of a number of factors, both environmental and physiological.

EPITOKY

Almost all polychaetes that become pelagic at sexual maturity undergo some degree of structural alteration into a specialized reproductive form (see Clark, 1961). This change is known as epitoky (Ehlers, 1864–68), and the transformed individual, an epitoke. Among the Nereidae this metamorphosis often achieves remarkable complexity, resulting in the formation of a heteronereis; but in most swarming polychaetes the changes are less pronounced and generally comprise histolysis of the body musculature and the digestive tube, as well as development of additional or modified setae. Such epitokous alterations have been described for several *Glycera* species (see Table I) and appear in mature specimens of *G. dibranchiata* as well.

External appearance of epitokes

In specimens completely or partially spent, the posterior two-thirds of the body is more collapsed and darker than the anterior portion. This appearance undoubtedly results from the retention of the proboscis, which provides more bulk anteriorly and lacks the dark pigmentation of the gut. Parapodia of epitokes are elongated and equipped with numerous setae. In the immature parapod shown in Figure 2A, the notopodial bundle consists of 7 or 8 simple setae, and the neuropodial bundle of about 22 composite setae. Two very short simple bristles are present just dorsal to the first neuropodial seta, but since this parapod comes from a worm with large gonads, these two unarticulated setae probably represent an initial stage in the development of the mature setal complement. In contrast, the notopodial bundle of the mature parapod (Fig. 2B) consists of 19 or 20 simple setae, and the neuropodial bundle includes, in addition to 37 composite setae, 8 dorsally placed simple ones. All of these bristles are noticeably longer than those of the atokous specimen but show no structural differences.

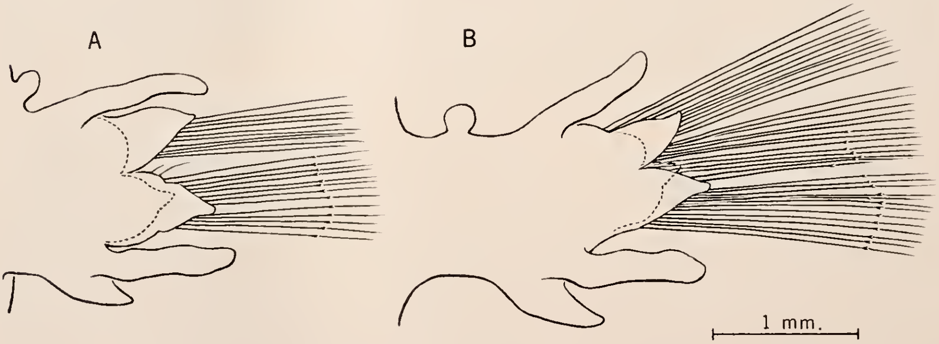

FIGURE 2. Parapod of (A) an immature specimen and (B) a swarming male. Both parapods are from the mid-body region and are shown in anterior view.

Although the epidermis of epitokes is reduced to a very thin layer, there is a marked increase in the activity of its mucous cells, especially in the parapodial lobes. A yellow-brown pigment, also present in other tissues of mature animals, is particularly abundant in the epidermis, where it often appears in the form of granular aggregates or minute, needle-like crystals. All the various chromogenic substances formed at epitoky probably are associated with degenerative phenomena and contribute to the coloration of mature worms. Klawe and Dickie report that male bloodworms about to spawn are a creamy color, while females are pale brown, and attribute these colors to the gametes showing through the thinned body walls. Though this may be the case in males, the eggs—being colorless—could hardly be responsible for a brown color in females. It is more likely that these differences stem rather from alterations of a metabolic nature. In the present study, color differences could be detected when mature males and females were compared in the laboratory, but it was very difficult to distinguish the sex of swarming worms in the field.

Internal changes

Epitoky is characterized by a drastic reduction in the thickness of the body wall and the diameter of the gut, with a corresponding increase in coelomic volume. Both muscle layers seem to be equally affected, but as the circular muscles are relatively thinner to begin with, this layer virtually disappears at epitoky. Since serpentine movements are executed primarily by longitudinal contractions, the difference in thickness of the muscle layers may play a role in the altered swimming behavior previously mentioned. The suspensory muscles of the digestive tube and the acicular muscles are also attenuated, though to a less striking extent. There is little apparent structural difference between muscle fibers of epitokous and atokous individuals; some vacuolization can be detected in the former, but this has occasionally been observed in immature worms as well. There is no marked invasion of the musculature by phagocytes, and in general, the epitokous condition seems to result more from atrophy than true sarcolysis.

The gut of mature animals is much reduced in diameter, with its shrunken mucosal layer appearing spongy and containing a granular yellow pigmentation. The columnar cells of this layer are apically disintegrated and have pycnotic nuclei. A scattered amorphous material occurring in the lumen probably consists of cellular debris. Despite these degenerative changes, the digestive tube, including proboscis and jaws, is entire.

The beginning of epitokous modifications in musculature and intestine appear in an ovigerous female collected 16 days before the first swarm. Since these changes are not yet visible in specimens that have just started to release gametes into the coelom, atrophy of the adult tissues may be related to metabolic requirements of the reproductive cells. Parapodial modification begins considerably earlier, before gametes appear in the body cavity. According to Støp-Bowtiz (1941), a similar sequence is found in *G. lapidum, G. alba* and probably *G. rouxii*, whereas in *G. capitata* it seems to be reversed, with degenerative changes preceding parapodial modification.

The coelomic epithelium and the septa are not affected by epitoky; nor do the segmental organs show any great change, although their protonephridial portions may be somewhat hypertrophied. The irregular black masses so numerous in the body fluid of mature worms are generally regarded as products of tissue breakdown, but their origin remains unknown. They are made up of clumped coelomic cells containing a greenish-brown granular substance that resembles the finer granulation found in red blood cells at earlier stages of maturity. Since these corpuscles are known to contain hemoglobin (Salomon, 1941), it seems possible that the greenish-brown pigmentation may be at least partially derived from decomposition of this molecule. Raphaël (1933) proposes that hemoglobin destruction and elimination takes place in the languettes attached near the jaws and projecting into the proboscidial coelom, but does not suggest how the compound is transported to these structures. Since the reduced languettes of epitokes are not particularly pigmented, it seems unlikely that they play a significant part in hemoglobin destruction during sexual metamorphosis.

The achievement of maturity in bloodworms is further accompanied by changes in the saccular apparatus of the brain. It has previously been suggested (Simpson, 1959) that this structure performs a secretory function, and present observations tend to support this hypothesis. In several swarming specimens the hyaline fiber, at its junction with the cuticle on the dorsal surface of the prostomium, forms a distinct opening through which an amorphous material passes to the exterior (Fig. 3). Although this is not clearly shown in all the swarming worms examined, no sign of such an opening has been found in either the animals studied in the earlier investigation or the non-swarming specimens of the present study. It seems probable, therefore, that some type of material elaborated by or stored within the saccular apparatus is released to the exterior at the time of swarming. Histochemical tests have not been performed, but the staining reaction of this material resembles that of mucus in epidermal goblet cells, and it may well be a mucopolysaccharide similar to that previously demonstrated for the saccular apparatus. The possible significance of such an external secretion is indicated by Clark's (1961) suggestion

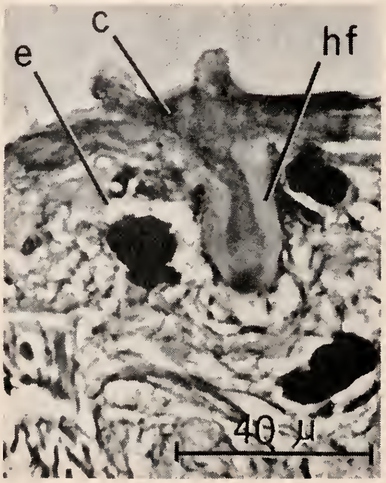

FIGURE 3. Section through the epidermis (e) on the dorsal prostomial surface of a swarming epitoke, showing the juncture of the cuticle (c) and the hyaline fiber (hf) from the saccular apparatus in the brain. The darker material within the hyaline fiber passes through the cuticle to the exterior.

that neurosecretory hormones released into the water might act as coordinating factors in the spawning of some nereids.

Other modifications of the saccular apparatus also suggest an enhanced activity of this structure in worms approaching maturity as well as those fully matured. Such alterations include an increase (rarely, a decrease) in size or number of sacs, a dilation and greater convolution of the filaments, and an enlargement of the vesicles. But since these changes do not occur consistently, and since the original description of the saccular complex is based on specimens from New England, the possibility exists that such changes may represent modifications indigenous to the Solomons population and not necessarily related to reproductive functions.

Epitoky and swarming

There is considerable variation in the degree to which mature specimens are affected by epitoky, and even among worms swarming at the same time, some individuals show far less atrophy than others. Furthermore, several animals in which maturity had been induced by an elevated temperature failed to spawn even though the gametes seemed to be ripe and epitokous manifestations were more pronounced than in any of the swarming specimens. It appears that of itself epitoky does not determine the time of swarming, but that such behavior can be elicited from animals that have undergone varying degrees of metamorphosis.

The epitokous characteristics observed here are of the same type as those described for other *Glycera* species. Although in the majority of other species degeneration, especially of the proboscis and gut, is reportedly far more severe, the degree of atrophy appears to be variable in these cases also. For example, Gravier and Dantan (1928) note an exceptional swarmer of *G. tesselata* in which the proboscis is intact, and Arwidsson (1898) describes a similar instance for *G. alba*. It is possible that the November swarming observed at Solomons occurred somewhat earlier than usual, before metamorphosis had reached its peak, or, more likely, that other species as a rule achieve more pronounced epitoky before swarming than does *G. dibranchiata*.

In view of these changes at maturity, it is generally assumed that glycerids do not survive swarming. For *G. dibranchiata,* Klawe and Dickie report that the occurrence of "ghost" worms, *i.e.* the remains of dead worms, was inversely proportional to the abundance of mature individuals. No "ghosts" were found during the present study, but since swarming took place in areas not exposed at low tide, the presence of such remains would be difficult to detect. Although spent worms placed in running salt water showed movement when handled some four or five days later, they underwent gradual deterioration and in about a week's time resembled "ghost" worms. However, since the degree of atrophy at swarming appears to be variable, it is conceivable that some individuals may be able to recover after shedding.

All of the field work involved in this study was made possible through the courtesy of the Chesapeake Biological Laboratory of the Natural Resources Institute, University of Maryland. I am greatly indebted to the Laboratory for the use of its facilities, and to the many staff members who provided valuable information and assistance during the investigation.

SUMMARY

1. This report is based on field observations made at Solomons, Maryland, between June, 1960, and February, 1961, and on histological examination of material collected during this period. It appears that bloodworms breed twice a year at Solomons: certainly during fall and very likely in late spring or early summer as well. Gametogenesis probably requires close to a year for completion. Both temperature and seasonal plankton variation are suggested as factors that may influence the timing of reproductive activity. There is reason to suspect that bloodworms also spawn biannually in the Maritime Provinces and Maine, but conclusive evidence is not available.

2. Swarming occurred mostly over shallow water, during late afternoon on November 5–8, 1960. It covered a moderately large area, but individual worms were widely dispersed. Data suggest that the onset of swarming may be coordinated with tidal conditions. Shedding is by dehiscence, through the posterior end, and is apparently an automatic process initiated by serpentine swimming movements that differ from the usual locomotion of immature animals.

3. Epitokes are characterized by atrophy of the musculature and alimentary canal, elongation of the parapods and increase in the number of setae. There are indications that the saccular apparatus of the brain releases a substance to the exterior during swarming. Although there is variation in the degree of atrophy attained at spawning, bloodworms apparently undergo less severe degenerative changes than other *Glycera* species.

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GAMETOGENESIS AND EARLY DEVELOPMENT OF THE POLYCHAETE GLYCERA DIBRANCHIATA[1]

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A previous paper (Simpson, 1962) has described fall swarming of the bloodworm, *Glycera dibranchiata* Ehlers, observed at Solomons, Maryland, during a study conducted there from late June, 1960, to early February, 1961. The same paper also discussed the probable breeding seasons of this species at Solomons, and presented data on the rate of gonad development, concluding that gametogenesis very likely requires close to a year for completion. A more detailed account of gametogenesis and an outline of development to the trochophore stage, obtained in the course of the same investigation, form the subject of the present report.

An early account of glycerid reproductive organs appears in Ehlers' (1864–68) original description of *G. dibranchiata,* and although inaccurate, has remained the only source of information on gonads in the genus. The embryology of *Glycera* is but slightly better known: Allen (1957) gives some data on the rate of early development in *G. ?sphyrabrancha,* Fuchs (1911) describes the young larval stages of *G. convoluta,* and observations on *G. dibranchiata* from fertilization to the trochophore are reported by Klawe and Dickie (1957). In the last two cases, as in the present one, attempts to maintain cultures beyond the trochophore stage were unsuccessful. This difficulty has also been alluded to by Wilson (1948), and it appears that further concentrated effort will be required to determine the complete developmental history of these annelids.

GAMETOGENESIS

Methods

The following observations are based largely on histological examination of about 70 specimens collected at Solomons (38°19′N., 76°27′W.) between June, 1960, and February, 1961. Animals were narcotized in magnesium chloride, fixed in Bouin's fluid and preserved in a mixture of 2 parts ethanol, 1 part distilled water and 1 part glycerine. Paraffin sections 4–7 μ thick were stained either with Ehrlich's hematoxylin and eosin, or by a modified procedure using Gomori's chrome-alum hematoxylin, recommended as a chromosome stain by Melander and Wingstrand (1953). Schiff's reagent, prepared by several different methods, consistently gave either weak or negative Feulgen reactions, and this technique was discontinued. Some attempt was made to study in more detail the chromosomal changes associated with gametogenesis by making squash preparations of gonads; results, however, were entirely unsatisfactory, probably because the material had been fixed and stored for several months. Gonads of fresh worms purchased at a bait store gave promising squash preparations with La Cour's (1941) aceto-orcein method, but this phase of the study was not pursued further.

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[1] Based on portion of a dissertation submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy at the Catholic University of America, Washington, D. C. Contribution No. 201, Chesapeake Biological Laboratory, Natural Resources Institute of the University of Maryland. This investigation was carried out during the tenure of PHS Predoctoral Fellowship BF-9242-C1 from the National Institute of Neurological Diseases and Blindness, and GF-9242-C2 from the Division of General Medical Sciences.

Gonads

Glycera dibranchiata is dioecious, with segmentally paired gonads beginning in the region of segments 45–47 and extending posteriorly for approximately 60 segments. Each gonad arises as an outgrowth from connective tissue at the lateral edge of the ventral longitudinal muscles, where the parapodial cavity opens into the general coelom. Between the gonad and the longitudinal muscles lies the origin of the ventral acicular muscle (Fig. 2), which passes dorso-medially from this point and inserts on a connective tissue pad surrounding the bases of the two parapodial acicula. From the same connective tissue pad other acicular muscles also radiate to their various origins on the body wall (Fig. 1); thus, the space into which the gonad grows is limited dorsally by the acicula, and anteriorly, posteriorly and medially by the acicular muscle bands.

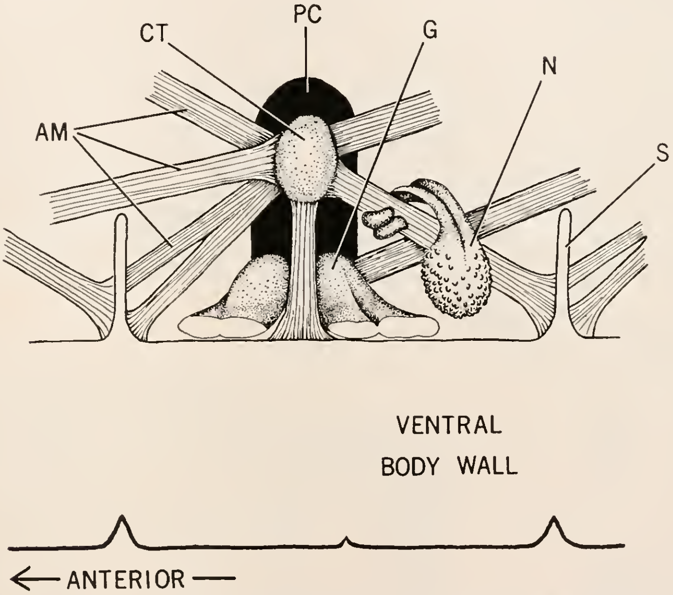
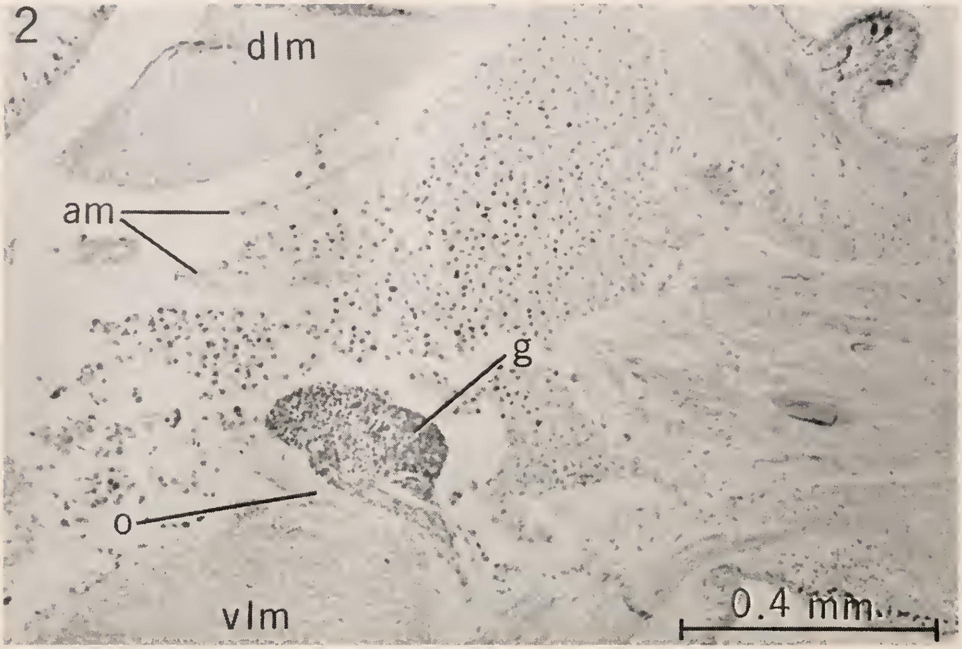


FIGURE 1. Diagram of the ventral half of one body segment, viewed from the median plane. Not to scale. AM, acicular muscles; CT, connective tissue pad around bases of the acicula; G, lobe of gonad; N, nephridial complex; PC, parapodial cavity; S, septum.

The initial gonial swelling gradually enlarges, extending further toward the coelom and occasionally a short distance into the parapodial cavity as well, but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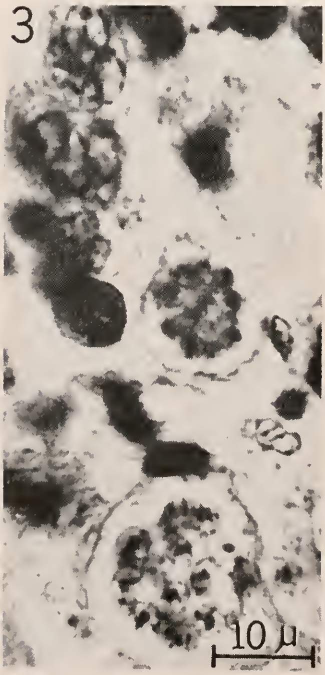

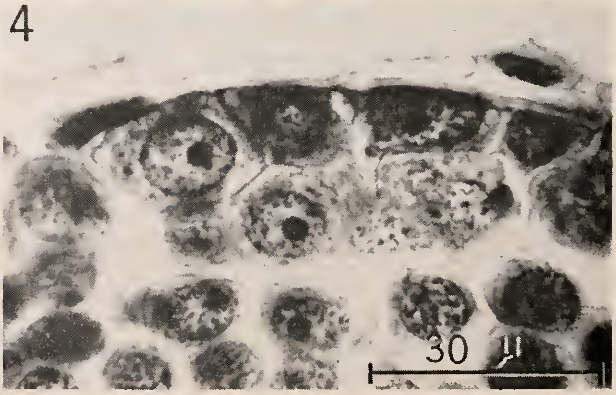

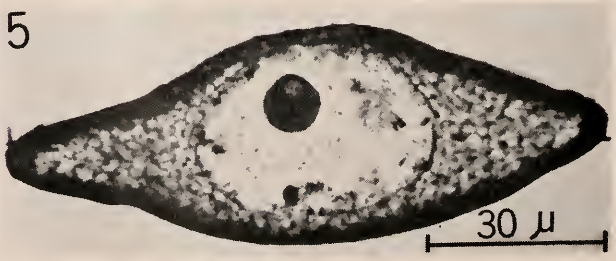

FIGURE 2. Transverse section through a mid-body segment, showing the position of the gonad (g). Part of the intestine appears in the upper left corner, and the dorsal cirrus of the parapod in the upper right corner. am, acicular muscles; dlm and vlm, dorsal and ventral

remains attached to its point of origin by a broad, compact stalk, which penetrates into the underlying connective tissue (Fig. 2). The nephridial duct from the preceding segment usually passes near or through this region of the gonad before turning laterally to the small nephridial pore just below the parapod. As the gonad expands toward the coelom, it becomes dorso-ventrally flattened between the acicula and their muscles, and eventually it pushes out between these muscle bands to form irregular lobes projecting into the body cavity. Since the degree of lobulation attained is subject to individual variation, no average dimensions can be indicated; maximum cross-sections range from 70 × 55 μ for very small gonads to 420 × 360 μ for large ones.

In dissections, large gonads can at times be confused with the nephridial complexes, which are also segmentally paired. The latter organs have the same general structure as those described in other *Glycera* species (Goodrich, 1898; Fage, 1906), but instead of being attached to the anterior face of the septum, each nephridial complex is connected only to the posterior acicular muscle (Fig. 1). The distal end of the ciliated organ makes a turn around this muscle, and the nephridial duct continues down the muscle to the body wall. Thus the main portion of the nephridial complex, consisting of the phagocytal sac and the protonephridium with its solenocytes, projects freely into the coelom and is occasionally found lying between the acicular muscles in the region of the gonad. In live specimens, the nephridial complex has a yellow-green tint readily distinguishable from the light pink of the reproductive organs.

The gonads of *Glycera* have previously been described by Ehlers (1864–68), but his account is somewhat confusing, since it is not entirely clear whether the description pertains wholly to *G. dibranchiata* or also includes *G. capitata.* His description of the ovaries (pp. 697–700) seems to be based on examination of epitokous females of *G. capitata,* yet the figures referred to are all labeled *G. dibranchiata.* These structures certainly bear little resemblance to the gonads described here; neither the "grape-like clusters" nor the simpler, fiber-like ovaries that Ehlers described have ever been observed in the present study. Besides, the females examined by Ehlers must have been far advanced in sexual maturity (as indicated by their pronounced atrophy and the masses of eggs in their body cavities), and according to the present observations, these specimens should have had no remaining gonad tissue whatsoever. On the other hand, the description of what Ehlers believed to be the testes (p. 700) is explicitly based on a specimen of *G. dibranchiata* and does agree with the present findings.

The matter is further complicated by Lubischev (1924), who states that Ehlers' illustrations of ovaries represent exactly the multiple nephridial complexes found in *G. capitata.* Since these illustrations are indicated as *G. dibranchiata,* Lubischev

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longitudinal muscles; o, origin of ventral acicular muscle. Melander-Wingstrand's modified chrome-alum hematoxylin (CAH).

FIGURE 3. Gonadal cells with basophilic material enclosed in separate vesicles (bottom and center of figure). Two cells at the upper left show the basophilic substance distributed around the periphery of each vesicle. Melander-Wingstrand's CAH.

FIGURE 4. Young oocytes at the surface of the gonad, shortly before they are released into the coelom. Gomori's CAH, phloxin.

FIGURE 5. Section through an oocyte shed spontaneously in the laboratory. Ehrlich's hematoxylin, eosin.

concludes that similar multiple nephridia are present in this species also, and that Ehlers must have mistaken them for ovaries. This conclusion, however, is incorrect, for *G. dibranchiata* has only two nephridial complexes in each segment, and these organs distinctly differ from the structures Lubischev found in *G. capitata*. It appears therefore that the "ovaries" described by Ehlers were in fact nephridial complexes of *G. capitata*, the legends to his figures notwithstanding. His misinterpretation was undoubtedly due to the presence of eggs in these structures, but it is not at all unusual to find gametes in the sac of the ciliated organ, which is closely associated with the nephridium proper (see Goodrich, 1945). Ehlers' description of the segmental organ, although not entirely accurate, indicates that he did recognize the nephridial complex in *G. dibranchiata*.

Early gametogenesis

The gonad first appears as an aggregation of cells embedded in connective tissue near the base of each parapod. The origin of these cells is uncertain, but they resemble the small, apparently amoeboid, basophilic cells scattered throughout connective and muscular tissues. The clustered gonial cells enlarge and proliferate upward, producing a bulge beneath the coelomic epithelium, which forms an investing membrane around the projection. From this early stage onward, the gonad is marked by the presence of relatively large (about $20 \times 11\ \mu$) oval cells containing scattered concentrations of chromatin. These cells are somewhat localized toward the interior of the gametal mass and in larger gonads occasionally seem to form a core extending from the stalk into the central region of the organ. There is, however, no sharp division into cortical and medullary zones, and the cells may occur in other parts of the gonad as well.

The chromatin inclusions within the cell vary in size and density, larger ones generally being more diffuse, with indistinct outlines, and smaller ones more compact and well defined. Their basophilia also varies with the degree of concentration, but never becomes very intense. In some cases, each inclusion appears to lie in a separate vesicle, giving the distinct impression that the larger body consists of a number of minute cells (Fig. 3). From this condition there follows a series of stages in which the basophilic material is distributed around the inner surface of each compartment, the individual vesicles coalesce, and the whole structure takes on the appearance of a cell with a large nucleus surrounded by a thin layer of cytoplasm. The significance of these cells in the gametogenetic process is not clear. In some aspects they strongly resemble stages of orthopteran spermatogonial divisions, in which individual chromosomes are separately compartmentalized (*e.g.* Wenrich, 1916; Rao, 1934), and it is possible that a similar situation occurs in *Glycera*.

The bulk of the gonad consists of developing gametes, which are at first closely packed and of uniform appearance, each cell containing a large reticulated nucleus surrounded by a small amount of cytoplasm. After a certain period, during which the gonad continues to grow, the germ cells enter a phase of nuclear activity and become more or less segregated into small groups, each containing cells at a similar stage of development. Although metaphase and anaphase configurations are relatively scarce, the nuclear chromatin shows various prophasic changes, undergoing condensation into thread-like filaments that thicken, become more basophilic, and form a compact tangled knot. Chromosome counts indicate that the diploid number probably lies between 30 and 40. Up to this point, ovaries and testes cannot be distinguished.

Oogenesis

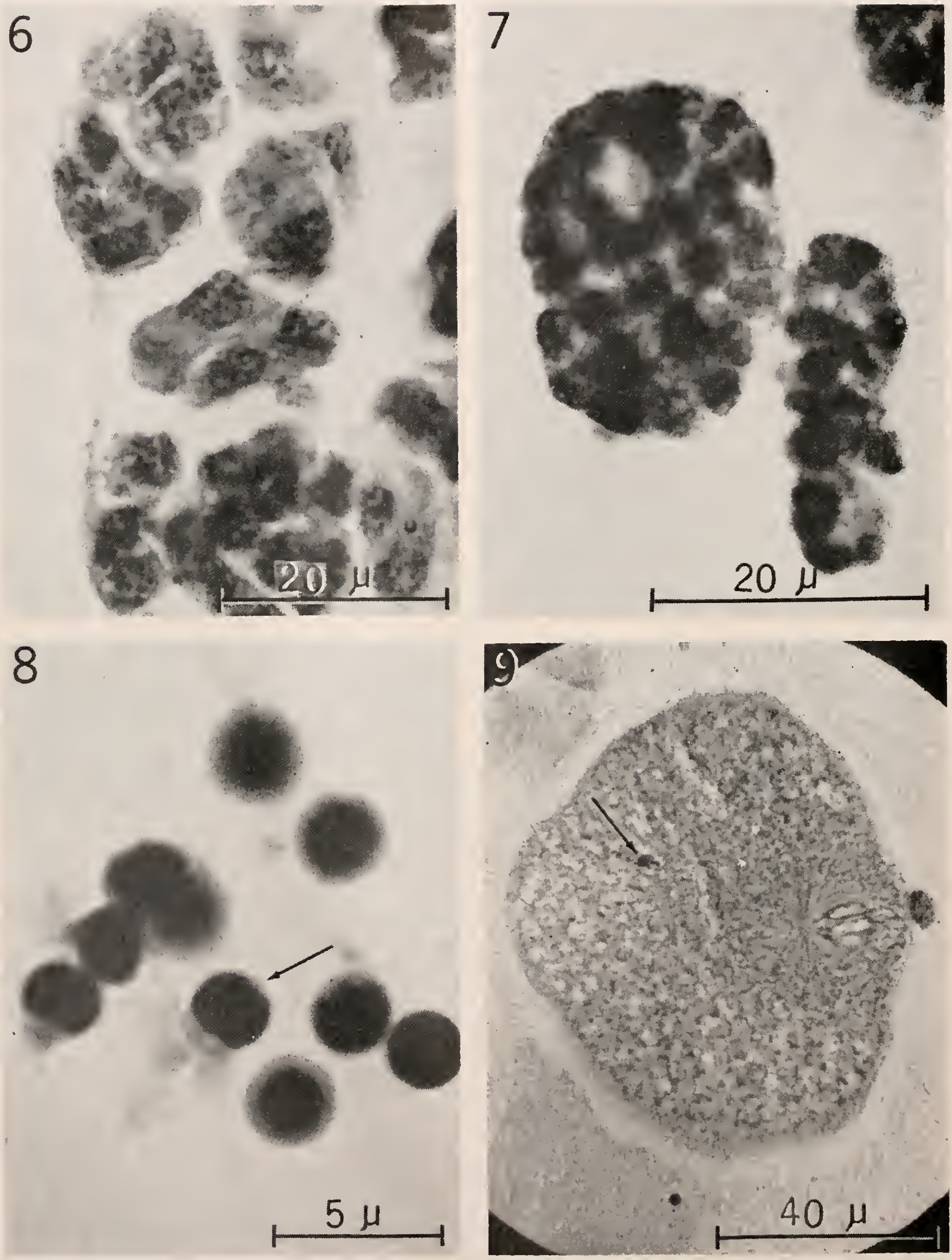
The first definite sign of sexual difference is the appearance of oocytes in the peripheral layers of the gonad, especially along its medial border. In these cells the dense chromatin mass relaxes into a diffuse network, a nucleolus appears, and the cytoplasmic volume begins to increase (Fig. 4). The stage at which the oocytes leave the gonad seems to vary. Apparently they are ready to be released upon reaching a diameter of about 28 μ, but occasionally the rupture of the epithelial covering is delayed, and they may grow larger before escaping into the coelom. As the oocytes are progressively released, the ovary decreases in size until no sign of it remains in gravid females. The oocytes complete their growth while circulating in the coelomic fluid, attaining an average diameter of 140 μ before being shed. No marked nuclear changes take place during this period, and since both polar bodies are formed after fertilization, it appears that the egg chromosomes rest in a diffuse state, having passed through early prophase of meiosis I in the gonad.

The ripe oocyte (Fig. 5) is a flattened sphere, bulging over the centrally placed germinal vesicle and surrounded by a thin membrane. Discoidal eggs have been described for other *Glycera* species (Fuchs, 1911; Allen, 1957) and are probably characteristic for the whole family. The colorless cytoplasm of the living egg is granular, with some spherical, refractile inclusions that may be small oil droplets; a thin cortical zone of fine granules is present. Oocytes fixed in Bouin's show a basophilic, alveolar cytoplasm. The germinal vesicle measures 50 μ in diameter and contains a diffuse acidophilic reticulum. The nucleolus is about 14 μ in diameter and appears to be double, consisting of a weakly basophilic, vacuolated portion that is cupped around a more homogeneous, acidophilic center.

Spermatogenesis

The spermatocytes similarly appear first in the periphery of the gonad, where they occur in small clusters of seven or eight cells (Fig. 6), which remain together after being released into the coelom. As in the female, the male gonad also becomes smaller and eventually disappears. When first released, individual spermatocytes have a diameter of 6 μ and contain a large nucleus with chromatin evenly distributed in the form of darkly staining condensations. The spermatocyte clusters develop into oval plates several cells thick, each plate consisting of approximately 30 or 40 cells and measuring about 16 × 9 μ (Fig. 7). Although division figures have not been observed, it seems likely that this increase in cell number is due to the maturation divisions. The nucleus of each spermatid is a rounded mass of chromatin, which appears to be honeycombed with minute vacuoles. The cytoplasm is reduced to a very thin layer and soon disappears entirely. With increasing condensation of the chromatin, the spermatids assume a somewhat triangular shape, while the sperm plates begin to loosen and break up. Apparently, spermiogenesis is completed after the individual spermatids have separated.

The mature spermatozoon (Fig. 8) is of the primitive type. Its spherical


6
20 µ
7
20 µ
8
5 µ
9
40 µ

Figures 6–9.

head is 2.5 μ across, bears a rounded acrosome, and is slightly flattened at the posterior border, where the four round elements of the middle piece are arranged around the base of the flagellum. Sperm remain active for at least six hours after being shed, but it is not known how long they retain the capacity for successful fertilization.

EARLY DEVELOPMENT

Methods

Embryological material was obtained during collection of swarming animals in November, 1960, when a male and a female were placed together in a jar. Although mortality due to polyspermy was high, the large number of eggs fertilized yielded an abundant supply of normally developing embryos. As soon as they were brought to the laboratory, the eggs were distributed into fingerbowls and washed five or six times. Thereafter the water in these bowls was changed every two hours, until swimming larvae appeared. Throughout the observations the cultures were kept in containers placed in a tray of running water to minimize temperature changes, and all water added to the cultures was first filtered through several layers of gravel and sand. Aeration was supplied by a small pump.

Several different methods were tried to maintain the larvae after swimming stages appeared. Some of the larvae were placed into cages made of small polyethylene containers of the type used to refrigerate food. The sides and covers of the containers were cut out to make large windows which were then covered with bolting silk, using a soldering gun with a smoothing tip to seal the material to the plastic. Four large corks attached to the corners floated the half-submerged cages in a large tank of running water. Unfortunately, larvae were able to escape through No. 18 bolting silk, and No. 20 became clogged so rapidly that within two days a microbial growth had flourished at the expense of the cultures. The same difficulty was encountered in using a current rotor device patterned after the apparatus of Galtsoff and Cable (1933; also Galtsoff, 1959). In this case, the rotating cylinder designed to keep the larvae in a small aquarium with a constant change of water was covered with bolting silk, which again clogged up rapidly. Better results were obtained with larvae placed in gallon jars half full of water, which was gently agitated by air passed through porous stones. The jars were covered with cellophane and fresh water was added as necessary to replace loss through evaporation. The relative success of these cultures suggests that the plunger-jar technique may have much to offer. Attempts to feed the larvae in different jars included the addition of diatoms (predominantly *Coscinodiscus;* also various pennate forms), a fine powder of dried clams, or substratum obtained from areas over which swarming took place. Despite the frequent occurrence of larvae with a bolus of reddish-brown matter in the gut

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FIGURE 6. Clusters of spermatocytes at the surface of the gonad, shortly before they are released into the coelom. Ehrlich's hematoxylin, eosin.

FIGURE 7. Two plates of spermatids free in the coelom. The larger plate is cut horizontally, the other vertically. Melander-Wingstrand's CAH.

FIGURE 8. Mature spermatozoa in the coelom of a swarming epitoke. The sperm in the center shows the acrosome (arrow), two elements of the middle piece and a short portion of the tail. Melander-Wingstrand's CAH.

FIGURE 9. Section through a fertilized egg in anaphase of the second polar division, showing the sperm head (arrow) penetrating into the center of the egg. Ehrlich's hematoxylin.

cavity, development ceased after the sixth day, and all the larvae died by the seventeenth day.

Rate of development

The observed rate of development at a water temperature of 12.5–14° C. is shown in Table I. Calculations are based from the time the swarming male and female were placed together; since both were shedding freely, it is assumed that fertilization occurred almost immediately. The times reported by Klawe and Dickie (1957) differ considerably from the present findings. This disparity arises mainly from the very slow rate of initial cleavages indicated in their report, and decreases in the more advanced stages. Thus the period from the 4-cell stage to the swimming embryo is about 12 hours in their report, and about 9½ hours in the present schedule, whereas the interval between swimming embryo and prototroch formation is approximately 10 hours in both cases. A difference in cleavage rate, however, is not unduly surprising, since Klawe and Dickie worked with a more northern population of bloodworms and made their observations over a wider temperature range (12–20° C.), using eggs shed and fertilized in the laboratory.

TABLE I

Rate of early development at 12.5–14° C.

Stage	Hours after fertilization
Polar divisions	<2
First division spindle	2½
Two-cell	3
Four-cell	4½
Eight-cell	5½
Swimming embryo	14
Trochophore	25

Fertilization and early cleavage

Germinal vesicle breakdown does not normally occur until fertilization. The nuclear membrane of unfertilized eggs disappears after about 10 hours at room temperature, but this is a degenerative phenomenon followed by gradual deterioration of the eggs. Within two hours of fertilization, the oocyte rounds up to a diameter of about 100 μ, and the vitelline membrane lifts away from the cytoplasm to form a wrinkled fertilization membrane 1.8 μ thick. Evidently a block to polyspermy is established by this stage, and numerous adherent sperm are lifted by the rising membrane. No external jelly layer is formed. The cortical granules of the unfertilized ovum have disappeared by this stage, but whether they contribute to the formation of the fertilization membrane is unknown.

Both polar divisions take place while the sperm head is approaching the center of the egg (Fig. 9). One of the polocytes, and occasionally the second also, divides again. Male and female pronuclei fuse in the center of the egg, and the first division spindle is completed by 2½ hours after fertilization. The chromosomes remain vesicular throughout the first cleavage and fuse to form a lobulated nucleus in each blastomere. The nuclei round out and elongate as the centrosomes migrate in preparation for the second division, during which the chromosomes regain their basophilia and typical form. Cleavage is spiral. The first four blastomeres appear to be equal, and the micromeres of the eight-cell stage are but slightly smaller than the macromeres.

Pelagic larvae

Thirteen hours after fertilization, perfectly spherical embryos are found rotating at the bottom of the dish and soon swim up to the surface. Gastrulation, apparently by epiboly, is in progress within the following three or four hours. Young trochophores are about 115 μ in diameter and have a broad prototroch as well as a patch of long apical cilia. They show no definite reaction to light, but are strongly geonegative, concentrating at the very surface of the water. This behavior does not persist, and larvae three days old are evenly distributed throughout the upper layers of the water.

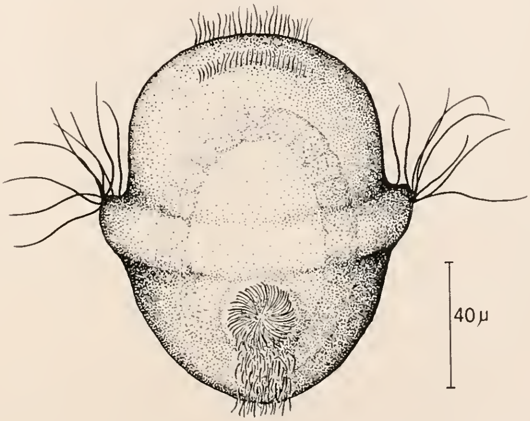

Figure 10. Ventral view of a six-day-old trochophore, drawn from a live specimen. Only the lateral portions of the prototroch are shown.

The six-day-old trochophore (Fig. 10) is about 120 μ long. In the legends to their illustrations of larvae, Klawe and Dickie give a length of 1.3 mm., but surely this must be a misplaced decimal point. At this stage, the larva has a complete equatorial ridge bearing two rows of well developed prototrochal cilia. An akrotroch is located ventrally, between the prototroch and the patch of short apical cilia. On the lower hemisphere, a band of cilia (neurotroch?) passes from the mouth to the posterior surface. The stomodeal opening is strongly ciliated, as is the whole surface of the gastric cavity, which seems to have in addition a ventral tract of exceptionally long cilia. When a food bolus is present, it is usually found in the anterior portion of the gut cavity, rotating counter-clockwise, as seen in dorsal view under the microscope. The gastric epithelium consists of large cells, many with vacuoles and granular inclusions. A distinct anus could not be found. The larva is not pigmented, nor does it possess eyespots.

Except for the lack of pigmentation, these larvae generally resemble the trochophores of *G. convoluta* described by Fuchs (1911). From Madras, Aiyar (1933) reports planktonic larvae which, on the basis of their similarity to the illustrations of Fuchs, he refers to the genus *Glycera*. Judging from Aiyar's figures, however, this similarity is not especially pronounced, and the presence of eyespots in the Madras larvae makes this generic classification questionable. Aiyar also describes (as *Eone*) nectochaetes of the goniadid *Glycinde*, in which eyes are present, and possibly his trochophores should be referred to this group instead of *Glycera*. Pelagic larvae of *G. alba* from Denmark have been described by Thorson (1946), but the trochophore is 400–450 μ long, has rudimentary anal cirri, and thus appears to be a later stage than any of the other trochophores reported. Thorson's record of metatrochophores and nectochaetes is the only one known for older larvae of *Glycera*. Treadwell (1936, p. 55) reports "two very young specimens of this genus, too immature for identification" taken in a plankton net at 800 fathoms off Bermuda, but gives no description. The specimens must have been relatively large, since the nets used were of No. 2 bolting silk (Beebe, introduction to Wailes, 1936).

Attempts made at Solomons to collect older larvae and post-larval stages were unsuccessful. A No. 20 net was used to take plankton hauls, both vertical and horizontal, at several different locations during the two months following swarming, but the few polychaete larvae obtained could readily be referred to *Polydora*. Examination of bottom samples produced no better results; all of these samples, however, were from very shallow areas, and cannot be considered truly representative. The smallest bloodworms found during this study were two specimens, 3 and 4 cm. long (fixed), collected in July, 1960. Klawe and Dickie (1957) report 3 cm. as the minimum for their observations, noting that these small worms were common in May and June. They also were unsuccessful in finding pelagic larvae or newly settled juveniles, and are inclined to think that the larvae spend only a short time in the plankton.

Much of this investigation was conducted at the Chesapeake Biological Laboratory of the Natural Resources Institute, University of Maryland, and I wish to express my thanks for the generous assistance rendered by members of the Laboratory staff.

SUMMARY

1. Information on gametogenesis in the bloodworm has been obtained by histological examination of material collected at Solomons, Maryland, in 1960 and 1961. The paired gonads begin in segments 45–47 and continue to about segment 110. They arise ventro-laterally, as retroperitoneal outgrowths near the opening of each parapodial cavity into the general coelom. Young oocytes and spermatocytes, the latter grouped in small clusters, are released from the gonads and mature in the coelomic fluid. Ripe oocytes are colorless, discoidal and about 140 μ in diameter; sperm are of the primitive type.

2. Insemination is followed by germinal vesicle breakdown, elevation of the fertilization membrane and both polar divisions. Pelagic stages appear within 14–20 hours and in a few days develop into unspecialized, apparently planktotrophic larvae. The further developmental process, including the period of metamorphosis and settling, remains completely unknown.

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CYTOLOGICAL STUDIES DURING GERMINAL VESICLE BREAKDOWN OF PECTINARIA GOULDII WITH VITAL DYES, CENTRIFUGATION AND FLUORESCENCE MICROSCOPY[1]

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It is becoming increasingly evident that subcellular cytoplasmic particles probably play an important part in the embryonic differentiation of the egg. The possible role of these entities in differentiation of the marine egg is aptly reviewed by Brachet (1957), Gustafson (1954), Raven (1958, 1961) and Shaver (1957).

One approach has been to identify and then trace the assignment of certain particulate groups during the early recognizable states in morphogenesis.

In the living egg innumerable investigations have used centrifugal forces to localize and subsequently identify the cell particulates by stratification. The early experiments by Lillie (1906, 1909) on the annelids *Chaetopterus* and *Nereis*, and of Morgan (1908, 1909, 1910) on *Arbacia* indicated that centrifugal force would stratify granules of yolk, pigment, mitochondria and other cytoplasmic particles according to their specific gravity.

In his classic paper on the egg of *Chaetopterus* Lillie (1906) observed the subcellular particulates in the living and fixed egg. With centrifugation of 1500 to 2000 rpm. he found that the endoplasm was stratified into a small grey cap, a clear band and a yellow hemisphere.

During germinal vesicle breakdown he noted the release of a residual substance composed of spherules and microsomes which he believed formed the grey centripetal cap. He also noted that the microsomes changed from an acid to a basic state as they moved into the cytoplasm and he followed their distribution during early cleavage.

More recent studies, using sucrose layering and much higher centrifugal forces, by Harvey (1939) on *Chaetopterus* and Costello on *Nereis* (1939, 1958) have contributed greatly toward identification of the various layers stratified in the annelid egg. Stratification in the egg of *Arbacia* (Harvey, 1941) was very similar to the annelid egg; in *Sphaerechinus granularis* the various strata obtained were almost identical to those obtained in the egg of *Chaetopterus* (Harvey, 1939). Thus a close comparison between the stratification of these eggs and the closely related eggs of *Pectinaria* seemed warranted.

Other observers have used intravital dyes in combination with centrifugation to identify cell particles in living eggs (Lucké, 1925; Harvey, 1941; Iida, 1942; Monné, 1944; Tweedell, 1960b).

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Further investigations have demonstrated a close relationship between certain vitally stained particles, such as the mitochondria, and cleavage activity of the egg. This linkage has been established in eggs of the sea urchin by Iida (1942) and Kojima (1959a), in *Urechis unicinctus* (Kojima, 1959b) and in the mollusc, *Spisula solidissima* by Rebhun (1959, 1960).

One role of mitochondria in differentiation of the ascidian eggs, *Beroë ovata* and *Phallusia mamillata,* has been verified. Here segregation of the mitochondria during development has been traced with Janus green. Subsequently, the mitochondria were found to be involved in the formation of the ciliary plates of the larvae. Significantly, the localized activity of the mitochondrial enzymes, cytochrome oxidase and succinic dehydrogenase, has been correlated with the Janus green staining of the mitochondria (Reverberi, 1956, 1957a, 1957b).

The pale yellow eggs of *Pectinaria gouldii* are extremely useful for cytological studies of this type because of their unusual transparency and relatively small amount of yolk. As a result, cytoplasmic and nuclear particles remain visible during development of the living egg.

In order to recognize and chemically identify the many particulates in the egg protoplasm of *Pectinaria*, numerous vital dyes and vital fluorochromes were applied to the living egg both before and after germinal vesicle breakdown. In addition to observations on these eggs, other eggs were centrifuged to separate and identify particles by their stratified position and staining characteristics. Further cytochemical tests on whole fixed eggs helped verify these findings.

Materials and Methods

The marine polychaete annelid, *Pectinaria* (*Cistenides*) *gouldii* Verrill, after Hartman (1941), is found in the mud-sand flats beneath shallow water beyond the low tide mark. This fascinating worm lives in a beautiful cone-shaped tube constructed of fitted sand grains, one grain in thickness, that are cemented together with secretions from the cementing organ (Watson, 1928).

Specimens for this study were obtained from the Woods Hole area by the Marine Biological Laboratory Supply Department. In the laboratory they were kept either in a large container of rapidly running sea water or in a shallow tray of sand that was submerged under running sea water. The animals usually burrowed with the head downward, as in nature, and the tapered end of the tube extended above the surface, especially at night. The adults did not survive long in the laboratory once they were removed from their tubes.

Prior to shedding, the eggs of *Pectinaria* are freely suspended within the coelomic fluid where they constantly shift back and forth with the pistonlike movement of the adult female. Many developmental stages of the oocyte are also present in the fluid. Following mechanical stimulation of the female within the tube, the coelomic fluid and eggs are vigorously shed into the sea water where the mature eggs continue maturation with breakdown of the germinal vesicle (GV). In the male, sperm packets are released in an identical fashion.

Eggs so obtained were studied immediately after shedding, with the bright field and dark field microscope. Other egg aliquots were shed directly into vital dyes prepared in sea water to stain various particulates. Alternatively, eggs were dyed with vital fluorochromes and observed under the fluorescence microscope.

Wherever possible, the specific vital dyes used were designated according to the revised color index numbers (CI).

Application of a single vital dye to the living egg often resulted in staining of more than one constituent of the protoplasm. For this reason both the untreated and dyed eggs were centrifuged in order to stratify and differentiate between identically dyed particles. Thus particles could be classified from their relative stratum after centrifugation and from numerous cross-checks with different dyes or combinations of microscopy.

Because of the preparatory time, the mature eggs had already undergone GV breakdown at the end of centrifugation but immature eggs were useful for studying particle distribution in pre-breakdown stages. Immature eggs did not undergo germinal vesicle breakdown on contact with sea water.

The eggs were layered over fresh sucrose (0.75 molar) in plastic centrifuge tubes, in the proportion of one-fifth sucrose to four-fifths eggs and sea water. The tubes were then placed in an ice bath prior to centrifugation. The eggs were centrifuged either in a refrigerated centrifuge (0° C.) at 3,000 to 4,850 *G* for one hour, in a high speed centrifuge at 17,000 *G* for 30 minutes at 4° C., or in an insulated preparatory ultracentrifuge (6° C.) at 33,000 *G* for 12 minutes. This enabled much sharper separation of granular bands than centrifugation at room temperatures.

After centrifugation, the eggs were immediately placed in an ice bath until the time of observation since lack of refrigeration allowed rapid "return" of the stratified components.

For all cytological observations the eggs were transferred in a small drop to the polished depression of a thin micro culture slide. A coverslip was then placed over the well so that the drop was in contact with the depression and the coverslip.

The light source for both standard dark field and fluorescence microscopy of the living eggs was a mercury vapor lamp (G. E. H4AB). The Osram HBO 200W bulb was found too intense for extended observations of living eggs.

In dark field observations a noviol "O" filter (Corning no. 3060) was used as an exciter filter at the light source to remove most ultraviolet, and a distilled water cell or an infrared cut-off filter (Kodak no. 301) for absorbing the infrared.

For blue light fluorescence (dark field) the infrared cut-off filter and a violet exciter filter (Corning no. 5113) were coupled to one of two barrier filters, a yellow shade (Corning no. 3486) filter which permits observation of fluorescence above 5100 Å or a noviol "A" filter (Corning no. 3389) that transmits down to 4200 Å.

Ultraviolet-induced fluorescence (dark field) was produced with either a blue purple ultra filter (Corning no. 5850), a red purple corex A (Corning no. 9863) or a Wratten 18A filter (Kodak). The same barrier and heat filters were used. Selection of filters for fluorescence depended upon the properties of the particular fluorochrome used.

A standard binocular microscope was equipped with a front surfaced aluminized mirror. Special funnel stops were used in the 20 ×, 40 × and 97 × objectives for all standard dark field and fluorescence observations, to reduce haloing from the relatively thick eggs.

Certain cytochemical reactions cannot be shown with vital dyes. In order to broaden the chemical tests and substantiate those obtained from the eggs stained *in vitro,* eggs were fixed at 30-second intervals from the time of shedding to 20 minutes after shedding. Similarly, eggs were centrifuged at 33,000 *G,* removed and fixed immediately at 0° C. with Kahle's fixative. The eggs were fixed directly on coverslips, using the double coverslip sandwich technique, and then stained with various cytochemical dyes.

Black and white photographs were taken on Panatomic X film with a Micro-Ibso attachment (Leitz). Color photographs of fluorescence and dark field were recorded on high speed Ektachrome (daylight) with a Zeiss attachment camera with movable prism. When the latter film was used for dark field photographs, a No. 3 gelatin filter (Kodak) was also used in conjunction with an infrared filter to absorb the low blue illumination.

The author wishes to acknowledge the helpful assistance of Mr. Christopher Watters whose unbiased suggestions were most useful during this research.

OBSERVATIONS

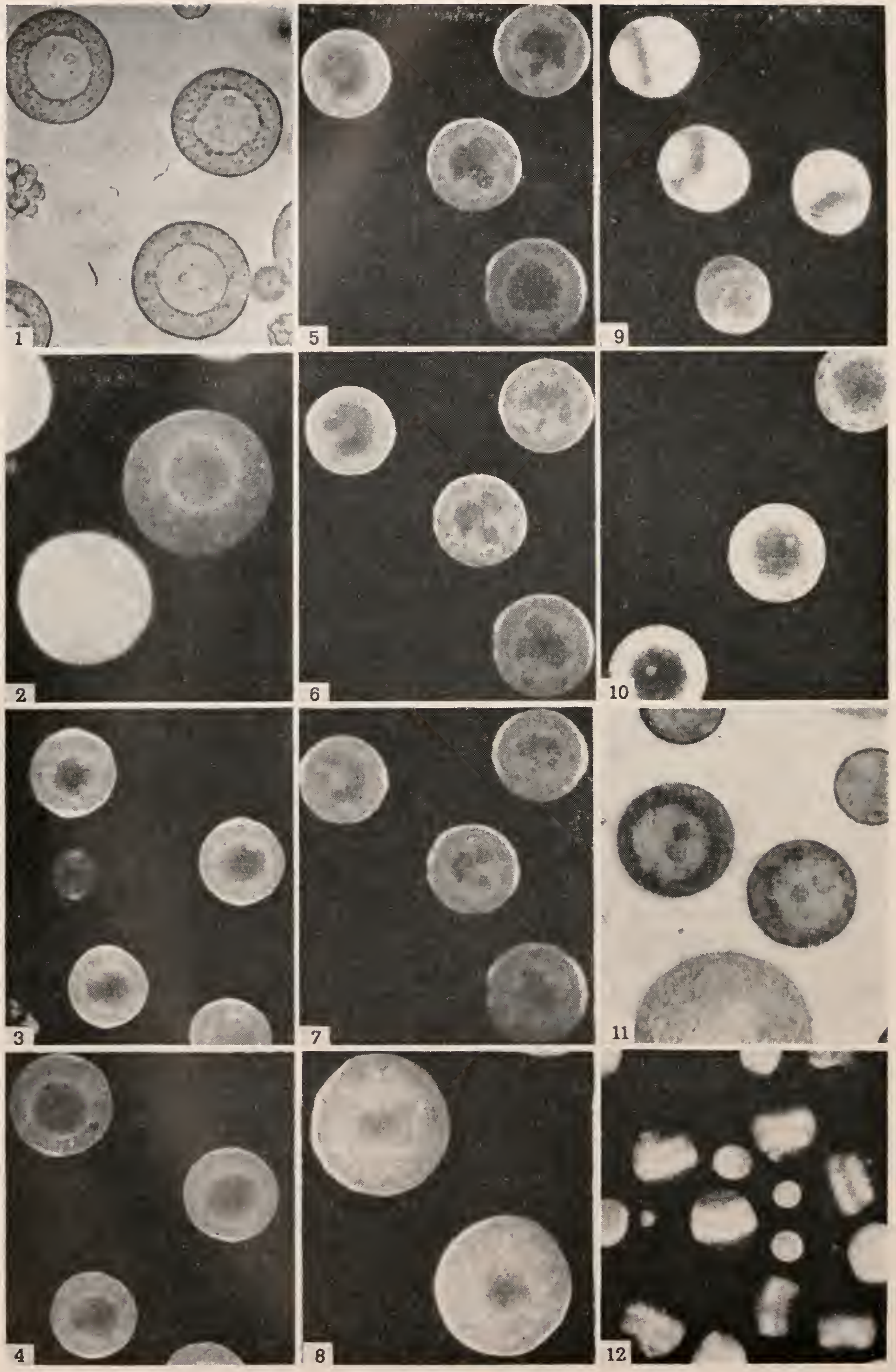
General appearance of the living egg

Prior to germinal vesicle breakdown the mature egg possesses a large germinal vesicle about two-thirds the size of the egg diameter. A prominent nucleolus, often double-lobed, is also present. The cytoplasmic ground color under visible light is pale yellow-green bounded by a yellow-green cell membrane. The egg measures 55 microns in diameter and is highly transparent although cytoplasmic particles are apparent (see Fig. 1).

Under dark field the particles are resolved into varying sizes of constantly agitated yellow-green granules. Smaller but extremely active silver-grey granules are more diffusely scattered throughout the cytoplasm. These also occur in irregular clumps around the nuclear membrane. The darker interior of the germinal vesicle contains a smaller number of similar granules. Sparsely scattered through the cortical cytoplasm are large yellow particles or clumps of granules.

Minute micro-villi project from the egg surface in a band covering the middle two-thirds of the egg. They are conspicuously absent in opposite polar regions and are much shorter in immature eggs. A living egg prior to germinal vesicle breakdown is seen under dark field in Figure 2.

Germinal vesicle breakdown. The eggs of *Pectinaria* at the time of shedding are slightly oval, flattened discs which almost invariably settle on their flattened surface. The germinal vesicle conforms to the oval shape but the outline is greatly scalloped during the first three minutes after shedding (Fig. 3). If the eggs are observed laterally, they resemble a dish standing on its edge. At 3.5 to four minutes post-shedding the germinal vesicle looses its scalloped edge and takes on a firm elliptical shape. Soon afterwards irregular clumps of highly motile, silver-white particles collect just outside of the membrane. The first indication of membrane breakdown is a wrinkling of the membrane that resembles the earlier scalloped appearance. Often, the nucleolus disappears at this time (Fig. 4). From six to ten minutes after shedding, depending upon the season, individual animals and temperature, the membrane opens, often on opposite sides. The silver particles flow through the gaps and form a temporary bridge, giving a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Figures 1–12.

transitory "hour glass" shape to the degenerating vesicle (Fig. 5). The initial breakthrough is followed by numerous irregular clumps of silver particles passing into the interior. The entire process of membrane dissolution lasts for 1.5 to three minutes (Figs. 6, 7). It is generally completed in all mature eggs 15 to 20 minutes after shedding. The nucleolus, if it has persisted, then gradually fades after membrane disappearance.

Soon afterwards, an indentation in the cell membrane forms on one side of the egg and beneath it a clear sac area develops. Here the first polar spindle forms

After disappearance of the germinal vesicle the egg has a small, dark irregular center surrounded by clumps of silver granules, numerous yellow granules of various sizes and points of fixed cortical granules (see Fig. 8).

Stratification of cell particles. Since dark field examination of the living egg had indicated the presence of several types of cytoplasmic particles, they were stratified by centrifugation. Living eggs were shed and allowed to undergo germinal vesicle breakdown. After cooling in an ice bath they were centrifuged for 15 minutes at 4000 *G* at 4° C. and examined immediately under dark field.

The cytoplasmic particles in *Pectinaria* consistently separated into three general regions, as seen in Figure 9. At the centripetal pole, an oval mass of fine, highly motile, silver white particles accumulated. This cap, referred to as zone A, consisted of small oil droplets at the apex and fine lipid particles.

FIGURE 1. Recently shed oocytes prior to GV breakdown. Unstained, reduced natural light.

FIGURE 2. A full size immature oocyte that did not undergo GV breakdown. Note the perinuclear ring of granules and the bilobed amphinuceolus. Dark field. 540 ×.

FIGURE 3. Newly shed (2.5 minutes) mature oocytes showing typical scalloped germinal vesicle. Dark field. 375 ×.

FIGURE 4. The same oocytes as in Figure 3 at 5.5 minutes after shedding. Germinal vesicle has become firm. 375 ×.

FIGURE 5. Mature oocytes just beginning GV breakdown at 9 minutes after shedding. The large, bright granular clumps are not involved. An amphiaster is forming on the extreme right. Dark field. 375 ×.

FIGURE 6. The same oocytes as in Figure 5 at 10 minutes after shedding. Notice the formation of cytoplasmic "bridges" across the old germinal vesicle. Dark field. 375 ×.

FIGURE 7. Germinal vesicle breakdown nearing completion at 12 minutes after shedding. Most of the silver white granules have flowed inward. Dark field. 375 ×.

FIGURE 8. Mature oocytes after germinal vesicle breakdown and 15 minutes after shedding. The oocytes remain in this condition until fertilization. Dark field. 540 ×.

FIGURE 9. Oocytes before and after GV breakdown that were centrifuged, showing stratification of cell particulates. In the post-breakdown egg is a crescent-shaped lipid zone (A), a hyaline zone (B), and a centrifugal granular zone (C). At the extreme centrifugal end is a flattened vacuolated zone. Dark field. 375 ×.

FIGURE 10. Fluorescing oocytes stained with acridine orange. The nucleolus is just disappearing at 10 minutes after shedding. Fluorescence: yellow, yellow green, and orange cytoplasmic granules; dark green GV; bright green nucleolus. 375 ×.

FIGURE 11. Immature oocytes stained with thionin demonstrating cytoplasmic granular uptake and slightly separated amphinucleoli, one staining reddish violet, the other remaining unstained. Mature oocytes are adjacent. Natural light. 750 ×.

FIGURE 12. Post-GV breakdown oocytes which were fluorochromed with acridine orange and centrifuged (15,000 *G*). The centripetal pole is up in most eggs (dark green fluorescence). The granular zone (C) consists of a centripetal bright yellow fluorescent band that is slightly separate from a more centrifugal band of yellow green and orange granules. The centrifugal pole fluoresces dark green. Filters: Corning 5113, 3486; Kodak 301. 375 ×.

Moving centrifugally a hyaline mid-region, designated as zone B, appeared black under dark field illumination. In the immature eggs, this area contained the germinal vesicle or nucleus of the oocyte. In this region also were scattered isolated clumps of fixed golden particles located in the cortical layer of the egg since they did not shift their position with centrifugation. These large golden, twinkling particles extended up into the silver lipid particles of the centripetal cap and later evidence showed that they reached to the extreme centrifugal end of the egg.

The lower centrifugal hemisphere of the egg, zone C, contained a heavy concentration of irregular sized yellow-green granules. Beginning near the equator of the centrifuged egg, where a diffuse layer of fine granules, the mitochondria, were detected, the granules of yolk increased in size and number toward the centrifugal end of the egg.

At the extreme centrifugal end of the egg, a vacuolated area containing a few dark granules was discernible.

The position of these stratified layers paralleled that found in the early investigations on *Chaetopterus* by Lillie (1906).

These three general zones were also seen in the immature and developing oocytes which still possessed a germinal vesicle. After centrifugation, a crescent-shaped cap of silver particles rested on top of the germinal vesicle at the centripetal pole. The germinal vesicle extended into the middle zone of the egg with the nucleolus displaced toward the centrifugal end of the vesicle. Sometimes a faint band of silver particles could be seen displaced halfway down within the vesicle or displaced around the nucleolus. Centrifugal to the germinal vesicle a U-shaped zone of mitochondria and yolk granules was found.

Staining the pre-breakdown egg

Earlier it had been found that living marine eggs could be fluorochromed with vital fluorescent dyes (Tweedell, 1959) and that the fluorescent inclusions persisted through subsequent cleavage and development. As a means of identifying these fluorescent cytoplasmic particulates, eggs of *Pectinaria* were freshly shed into a variety of vital fluorochromes so that the inclusions could be studied before GV breakdown. The general procedure for these dyes is outlined for acridine orange, a vital fluorochrome that produces metachromasia in living tissues. Acridine orange also has a specific affinity for DNA and RNA-proteins under controlled conditions (von Bertalanffy and Bickis, 1956; Tweedell, 1960a).

Eggs were shed into a 0.001% solution of acridine orange (CI 46005) in filtered sea water and allowed to stand for three minutes. They were then centrifuged lightly, the stain decanted off and the eggs washed twice in fresh filtered sea water to remove background fluorescence. This dye absorbs maximally at 4100 Å and thus most observations were made with a violet exciter filter that transmits maximally at 4100 Å and a yellow shade barrier filter plus an infrared filter. This combination gave a completely black background but provided green, yellow and red fluorescence. Substitution of a noviol "A" barrier filter allowed transmission of visible blue light that also permitted non-fluorescing components to be seen, a procedure useful when centrifuged eggs were viewed under dark field.

When eggs were fluorochromed as above, the cytoplasm fluoresced pale green and was filled with numerous yellow-green and orange granules of various sizes; the entire egg was enclosed by an orange fluorescent cell membrane (Fig. 10). The light yellow-green nucleolus, containing one or more non-fluorescing vesicles, appeared within the dark green germinal vesicle, which was surrounded by a perinuclear band of yellow-green granules.

About seven minutes post-shedding, the nucleolus disappeared and distinctive bright green fluorescent clumps of a constant size and number, presumably chromosomes, appeared within the germinal vesicle.

Following GV breakdown, the egg cytoplasm contained a scattered mixture of yellow, orange and yellow-green granules. About 15 minutes after breakdown, the bright yellow-green chromosomes reappeared in a tight knot near the center of the egg in the first maturation metaphase. These two observations were the first ones seen of the chromosomes in the living egg of *Pectinaria*.

Further substantiation of the identity of the chromosomes before and after GV breakdown came from the application of histochemical dyes to the fixed eggs. The same assemblage of chromosomes could be seen in the intact egg following the Feulgen reaction, staining with gallocyanin chrome-alum under conditions made specific for nucleic acid concentration (Lagerstedt, 1949; de Boer and Sarnaker, 1956, cited in Pearse, 1961) and with Galigher's haematoxylin.

The chromosomes were also very sharply defined in the fixed whole egg after they were extracted with trichloroacetic acid and stained with Schiff's reagent.

Secondary fluorescence was also induced in the pre-breakdown egg with neutral acriflavine (National Aniline). Since this dye absorbs at the same wavelength as acridine orange, the same filters were used. Acriflavine has been shown to have an *in vivo* affinity for intranuclear proteins (De Bruyn *et al.*, 1953). A basic dye, it combines with the phosphoric acid groups of the nucleic acids (Brachet, 1957).

Eggs were shed directly into a 0.001% solution of the dye in sea water. In the living egg bright lime-green fluorescent granules were evenly dispersed through the cytoplasm. Within the nucleus, the nucleolus fluoresced bright yellow-green. Large vacuolated spheres were also seen within the nucleolus. With the exception of the nuclear membrane, the interior of the nucleus, strangely, remained unstained.

The nucleolus. The nucleolus in the mature oocyte was a large single body with one or more eccentrically placed vesicles or vacuoles, differentiated by their general lack of staining affinity. The body of the nucleolus fluoresced lime green with acridine orange or acriflavine, dark green with Janus green and blue with toluidine blue in the living egg. In fixed material, the nucleolus was Feulgen-positive and stained dark purple with gallocyanin chrome-alum. The vesicles or vacuoles remained unstained in each case.

In the younger oocytes the vesicles occurred as epinucleolar buds but in the mature oocytes, they appeared to be intranucleolar vacuoles (Raven, 1958). Both types existed. The immature oocytes that still possessed nucleoli after high speed centrifugation (33,000 G) occasionally showed a telescoped chain of three, sometimes four vesicles.

In the less mature oocytes, the nucleoli usually appeared as double-lobed amphinucleoli (Wilson, 1925), one lobe larger than the other. At times, the nucleoli were separated but very often they were united. The principal nucleolus often had one or more accessory nucleolar buds attached to it.

The chemical differences within the amphinucleoli were apparent after staining in thionin, a basic vital dye that exhibits metachromasia. The smaller lobe generally stained deep reddish violet while the larger lobe remained perfectly clear (Fig. 11). In mature oocytes, the single nucleolus never became stained although this may have been a function of the short staining time before nucleolar breakdown.

The youngest oocytes, judged by their smaller diameters, generally possessed a greater number of small separate nucleolar-like bodies. No attempt was made to study the origin or development of these nucleolar bodies but it was noticed that the small nucleolar vesicles in the very young oocytes fluoresced bright red in contrast to the bright green of the main nucleolar body, after treatment with acridine orange.

Centrifugation of vitally dyed eggs

With the application of vital fluorochromes and other dyes to the pre-breakdown egg it became apparent that many granules of diverse shapes and sizes were scattered randomly throughout the cytoplasm. Many of these consisted of lipid and proteid yolk granules. However, even the common position of these which stained with different fluorochromes or dyes did not necessarily indicate that they were identical.

As a step toward resolving the mixture of cytoplasmic granules, the eggs were vitally dyed or fluorochromed after GV breakdown and centrifuged at 30,000 *G* for 15 minutes in a precooled head maintained at 4° C. throughout the run.

When the eggs fluorochromed in acridine orange were examined under blue light and a yellow shade filter, the principal fluorescence came from a heavy concentration of yellow or orange granules displaced toward the centrifugal pole in zone C. The rest of the egg appeared deep green (Fig. 12). The extreme centrifugal pole also contained an irregular agranular area that remained deep green.

In almost the exact center of the centrifuged egg, at the junction of the yolk granules and the empty mid-region, a tight knot of lime green chromosomes was often seen.

Substitution of a noviol A barrier filter revealed the silver blue cap of lipid granules in zone A. The deep green background of the hyaline zone changed to deep blue and the entire granular area in the centrifugal zone fluoresced violet-orange.

Proteid yolk and mitochondria. With more extensive centrifugation a second band of yellow-green granules was slightly separated from the main mass of orange yolk granules nearer the mid region of the egg. This distinct band appeared at various levels, depending upon the total centrifugal forces, but always rejoined with the main centrifugal mass of granules shortly after centrifugation. The fluorescence of these granules appeared to be identical to the perinuclear band of granules seen in the pre-breakdown egg.

After centrifugation the apparent homogeneity of the cytoplasmic granules stained with acriflavine also disappeared. A large concentration of yolk granules was again located in the heavy centrifugal end (zone C) of the egg. Under U. V. or blue violet light and a yellow barrier filter they fluoresced yellow green which became more intense nearer the upper end at the equator of the egg. However, with blue violet illumination and a noviol filter that permitted blue violet transmission, these granules were quickly differentiated into a more centripetal band of brilliant, yellow fluorescent granules and a lower centrifugal zone of bluish granules. Fluorescence in the more centrifugal part of the zone was masked by the stronger transmitted blue light. Here was another indication that two general groups of granules made up the large zone at the centrifugal end of the egg (Fig. 13).

The majority of the granules concentrated in the centrifugal zone of the egg consisted of proteid yolk granules of different sizes. These granules were generally readily stained with several fluorochromes and other vital dyes. Centripetal to and overlapping the yolk granules in the equatorial zone of the egg was another layer of granules that were often differentiated by color and particularly on the basis of their fluorescence. The earlier cited centrifugation experiments of Harvey (1939) and Costello (1939; 1958) on the annelid egg indicated that the fluorescent band seen with acridine orange and acriflavine, just centripetal to the main mass of yolk granules in the egg of *Pectinaria,* corresponded to the position occupied by the mitochondria.

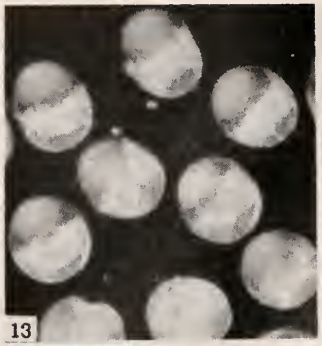
A separate yellow fluorescent equatorial band was also obtained after the application of two other distinctly different cytoplasmic fluorochromes, both used in the identification of fats (Popper, 1941). The first, thioflavine S (CI 49010) with a peak absorption at 3650 Å, was applied (0.0002%) to the living egg and observed under U. V. In the germinal vesicle stage the cytoplasm was filled with a mixture of yellow green granules. A separate perinuclear band of yellow green granules surrounded the pale green nucleus and a light green nucleolus.

After centrifugation, both the U. V. and blue light illumination showed yellow green granules confined to zone C in the centrifugal pole of the egg. When U. V. light was coupled with a noviol O filter permitting full spectrum fluorescence, a separate yellow green band of granules could be identified across the upper end of the yolk granules in zone C. The greater mass of proteid yolk granules centrifugal to the band appeared blue since the complex formed by the dye and the yolk granules absorbed light at a higher wave-length.

This distinction was not just the result of differences in intensity of fluorescence. A second fluorochrome, phosphine 3R (CI 46045), produced brilliant yellow fluorescence in a wide band of granules around the germinal vesicle. After GV disappearance, the vitally stained eggs were centrifuged and the whitish yellow granules were exclusively located in a thick band along the upper margin of the yolk mass in zone C. The rest of the granules did not fluoresce. This was the same area demarcated by acridine orange, acriflavine and thioflavine. It suggested that the difference in fluorescence was a qualitative measure of lipid content as well as particle size.

The lower centrifugal granular portion of zone C could also be sharply delineated by its fluorescence from the more centripetal fluorescent band with auramine O (CI 41000). This fluorochrome was very successfully used for dying the tubercle capsule (Richards and Miller, 1941) which is rich in neutral polysaccharides.

Application of a 0.001% solution of auramine O to the pre-breakdown egg produced fluorescence extending as a broad band of brilliant yellow cytoplasmic granules around the nuclear membrane.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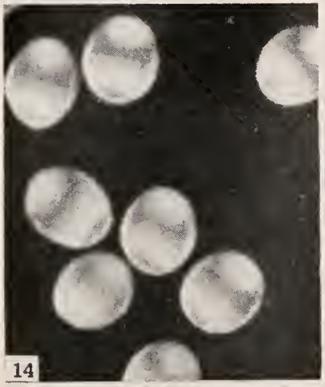
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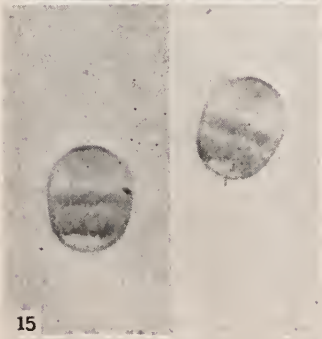
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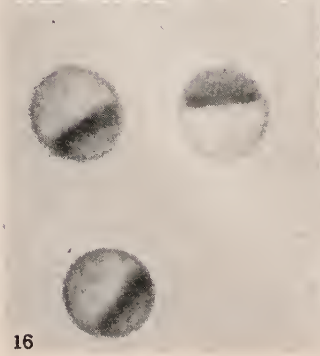
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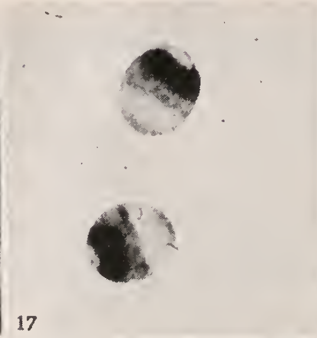
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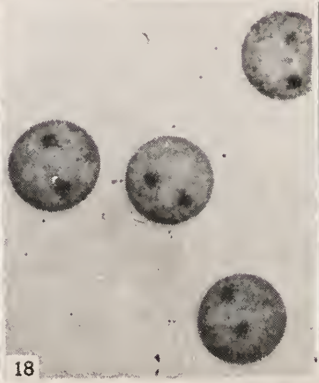
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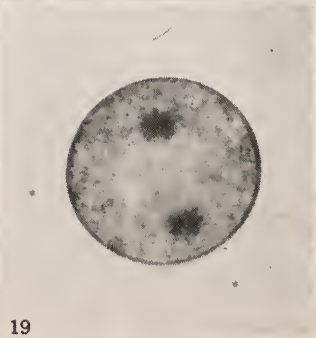
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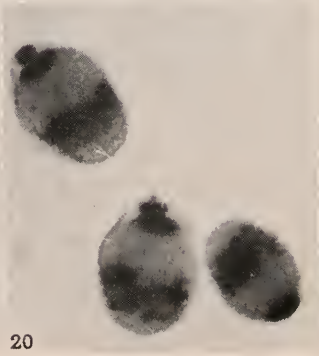
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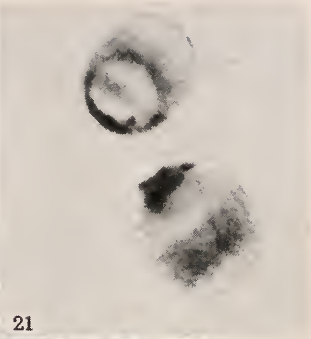
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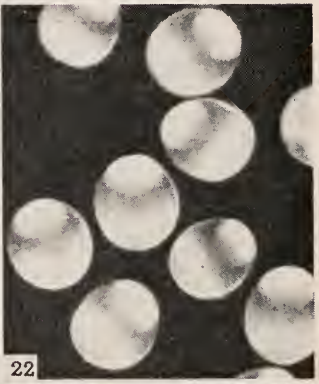
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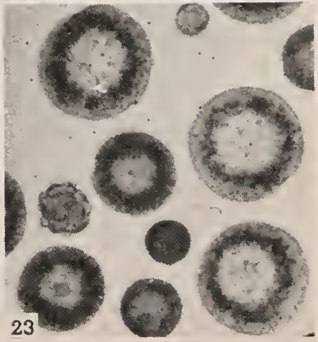
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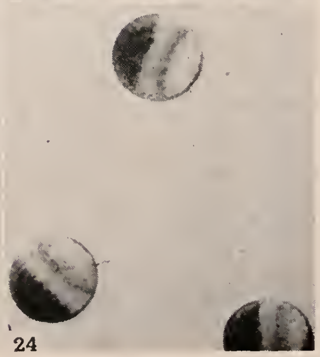
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FIGURES 13–24.

Following centrifugation, the yellow fluorescent yolk granules were only found in the lower centrifugal region of zone C. The previous fluorescent band that fluoresced with acriflavine, thioflavine, and phosphine appeared blue from trans-illumination. Only the proteid yolk granules were activated by the U. V. illumination (Fig. 14).

The centripetal band of mitochondria in zone C was also clearly defined after staining with other vital dyes. Both Harvey (1941) and Monné (1944) found that gentian violet (crystal violet) gave a very intense stain with mitochondria in the sea urchin egg.

After staining the eggs with a 0.0001% solution of crystal violet (CI 42555) for 30 minutes and subsequent centrifugation at 33,000 *G* for 15 minutes, a sharp blue band of granules appeared at the centripetal end of zone C. This corresponded in position to the band seen previously with the fluorescent dyes (Fig. 15).

A temporary light blue ring was also seen just below the lipid cap in zone A but this diffused rapidly into the lipid cap.

Mature eggs of *Pectinaria* were next shed into another vital thiazine dye,

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FIGURE 13. Post-GV-breakdown oocytes that were fluorochromed with acriflavine and centrifuged. The centripetal pole (upper left) contains blue illuminated granules. The lower centrifugal pole has a bright yellow band of fluorescent granules above the more centrifugal bluish-yellow granules. Filters: Corning 5113, 3060; Kodak 301. 375 ×.

FIGURE 14. Centrifuged oocytes that were fluorochromed with auramine and centrifuged. The oval centripetal zone appears blue. In the centrifugal zone only the lower proteid yolk granules fluoresce yellow. Filters: Corning 5850, 3389; Kodak 301. 375 ×.

FIGURE 15. Centrifuged oocytes stained with crystal violet. The lipid cap at the centripetal pole is surrounded by a band of light blue granules. Across the center is a blue violet granular band, slightly separated from the centrifugal yolk granules. 375 ×.

FIGURE 16. Centrifuged oocytes stained with Janus green. An intense layer of deep green granules lies just below the equator. More diffuse grey green granules extend to the centrifugal pole. 375 ×.

FIGURE 17. Centrifuged oocytes stained with Nile blue sulphate. The wide mass of dark blue granules at the centrifugal pole is slightly separated from an equatorial band of metachromatic (reddish-purple) granules. 375 ×.

FIGURES 18, 19. Post-GV-breakdown oocytes that were stained with Nile blue sulphate, showing metachromatic astral granules. Deep blue yolk granules are scattered through the blue cytoplasm. Natural light. Figure 18 shows eggs after recovery from centrifugation. 375 ×. Figure 19 is an uncentrifuged egg. 860 ×.

FIGURE 20. Centrifuged oocytes after GV breakdown. These eggs were fixed and stained by the Nile blue test. A deep red lipid drop and red violet lipid cap occur at the centripetal end. The hyaline layer is light blue. At the equator is a band of metachromatic granules and scattered purple granules. The centripetal zone contains deep purple granules. 500 ×.

FIGURE 21. Centrifuged eggs after GV breakdown, fixed and stained with Sudan black B. Black lipid droplets and a lipid granular cap located the centripetal end. A faint granular ring surrounds the lipid cap and a broad band of granules in zone C lies centripetal to the centrifugal pole. The immature oocyte on the right shows similar stratification plus a thin granular zone within the germinal vesicle. 500 ×.

FIGURE 22. Post-GV-breakdown oocytes stained with rhodamin O and centrifuged. The pink band of granules centripetal to the lipid cap is faintly detectable. Bright yellow green granules fill the centrifugal pole. Dark field. 375 ×.

FIGURE 23. Pre-breakdown oocytes stained with toluidine blue O. A heavy concentration of deep blue granules surrounds the nuclear membrane and grades out into the light blue cytoplasm. 465 ×.

FIGURE 24. Post-GV-breakdown oocytes stained with toluidine blue and centrifuged. A heavy collection of blue granules occurs at the centrifugal end. Just centripetal to the unstained lipid cap is a thin band of metachromatic granules. The hyaline layer is faint pink. 375 ×.

thionin (CI 52000), also used by Harvey (1941) for the sea urchin egg. This dye penetrated the living egg extremely slowly at all concentrations but did produce a faint lavender band of particles just outside the nuclear membrane. During GV breakdown, these particles collected along the indentations of the degenerating membrane. The particles became diffusely scattered throughout the cytoplasm after GV breakdown. Centrifugation failed to demonstrate any localized band of particles.

The immature eggs took up the stain readily, which first concentrated in the perinuclear granules and the nucleoplasm. As mentioned, the most effective concentration of the dye occurred in the nucleoli (see Fig. 11).

To further identify and localize the mitochondria, Janus green B (CI 11050) was applied to the living eggs. The specificity of Janus green for mitochondria has been well substantiated in a variety of marine eggs. For example, centrifugation experiments using Janus green as an index of mitochondria in eggs of *Ciona intestinalis* and *Phallusia mamillata* by Mancuso (1959), and La Spina (1958) all showed the mitochondria localized in the centrifugal half of the egg between the yolk and the centrifugal hyaline cap.

After eggs had been shed into a 0.001% solution of Janus green in sea water, diffusely scattered granules with a rather faint blue green coloration appeared throughout the cytoplasm. In immature eggs, there was a tendency for these granules to clump around the nuclear membrane in a perinuclear ring. After GV breakdown, they remained diffusely scattered in the cytoplasm.

Centrifugation of the eggs stained with Janus green generally showed that the blue green granules were confined to a slightly concave area in the centrifugal end of the egg. Rapid cross-comparison of the centrifuged egg under dark field with the stained egg under bright field demonstrated that the concentration of Janus green particles overlapped the heavy granular centrifugal portion of the egg. The cortex around the granular area remained unstained and well defined. In addition, strongly defined cortical granules were often seen to extend around the circumference of the egg.

The position of the displaced granules was not always consistent in the centrifuged egg. Often, a sharp, broad band of granules was localized across the equator of the egg while more grey green granules extended toward the centrifugal end. This heavy equatorial band always appeared in the centripetal portion of zone C, and in these cases corresponded to the position where the mitochondria usually stratified (Fig. 16).

Shaver (1957) found that Janus green was not satisfactory for demonstrating mitochondria in the intact cells of sea urchin embryos. Under phase contrast he observed they had a tendency to clump, indicating that a physical change took place in the granular elements after dye penetration. A similar phenomenon may have caused the inconsistent localization of mitochondria by Janus green in the living egg of *Pectinaria* after centrifugation.

The previous tests that differentiated the mitochondria from the proteid yolk suggested the use of Nile blue sulphate on the living eggs. Nile blue sulphate (CI 51180) is an oxazine dye used for histochemical differentiation of lipids. It is a commonly used vital dye and exhibits metachromatic properties even in the living egg. Monné (1944) had found that Nile blue stained the yolk platelets blue in the sea urchin egg. However, blastulae of the sea urchin were vitally stained with Nile blue sulphate by Gustafson and Lenique (1952) which enabled them to follow the number and distribution of mitochondria.

The eggs of *Pectinaria* were shed into a 0.001% solution and subsequent to GV breakdown, the cytoplasm gradually became light blue and the yolk granules became deep blue. In the immature eggs in which the nucleus had not broken down, an elliptical band of heavy purple granules eventually appeared around the intact nuclear membrane.

After centrifugation at 4160 *G* for 15 minutes, the post-breakdown egg showed a heavy concentration of deep blue granules at the centrifugal end. Toward the equatorial region of the egg, the granules of zone C formed a distinct band of reddish purple granules. This band occupied the same position as the band of granules seen with the previous fluorochromes. Under prolonged centrifugation at 33,000 *G* the band was distinctly separated from the yolk and remained in the mid region of the egg. The centripetal cap in zone A remained unstained (see Fig. 17).

Astral granules. In the mature egg the metachromasia induced by Nile blue sulphate did not become evident until after germinal vesicle breakdown and the formation of the first maturation spindle. Upon standing in fresh sea water after staining, many of the eggs exhibited two crescent-shaped groups of granules that accumulated around each end of the clear spindle area near the equator of the egg. These constantly agitated granules appeared about 45 minutes after shedding. Under visible light they appeared reddish violet, in sharp contrast to the uniform blue stained yolk granules. Their uniform position in all the eggs (Figs. 18, 19) suggested that they were associated with the astral rays at each end of the maturation spindle.

The same phenomenon was observed in the centrifuged eggs after the stratified granules had begun to return to their original position. Within three hours after centrifugation, identical groups of reddish violet granules were found in these eggs. In many instances they were grouped around the first polar body which often formed after centrifugation.

Extended observations of these eggs revealed that the astral granules originated from the equatorial band of reddish granules in zone C.

Nile blue test. Fixed centrifuged eggs were next submitted to the Nile blue histochemical test (Casselman, 1959) in order to verify the metachromatic staining seen in the living egg. The granules in the lower part of zone C consistently stained deep blue, the identical result found in the living centrifuged eggs. It also suggested that these granules were primarily proteid yolk since the oxazine component of Nile blue forms blue salts (Cain, 1947, cited in Casselman, 1959) with fatty acids and phospholipids. The more centripetal band of zone C was again filled with reddish purple granules. A non-acidic red reaction is created by the oxazone component with glycolipids and simple lipids. It seemed likely that these granules were rich in glycolipids.

A strong reddish purple reaction was also produced in the centripetal cap of zone A. Just above this cap, at the extreme centripetal end of the egg, one or more wine red lipid droplets collected after centrifugation, just within the cell membrane. Very often they coalesced into a single red lipid droplet. Scattered between the lipid cap in zone A and the centrifugal zone were sparsely scattered blue granules, apparently located in the cortex (see Fig. 20).

It is well established that fatty substances are found in oocytes of many different animals (Raven, 1958, 1961). These may occur as free lipids in the cytoplasm, as fatty yolk globules or they may be contained within cell components such as the mitochondria, Golgi bodies, etc.

One of the best cytochemical indicators of overall lipid distribution is Sudan black B. Since the dye is actually dissolved in the lipid, but not in water, it must be used on fixed material.

Centrifuged eggs were fixed in 10% formalin and 1% $CaCl_2$; the eggs were dyed with a 1% solution of Sudan black B in 60% triethyl phosphate (Casselman, 1959) and mounted in glycerin jelly (see Fig. 21).

At the centripetal end of the egg in zone A, the lipid cap stained black and was surrounded centrifugally by a light grey band of fine granules. Either within the lipid cap or just centripetal to it were one or two large intensely black droplets formed by free lipids.

The hyaline zone was clear except for prominent black isolated cortical granules extending the full length of the stratified eggs.

Moving centrifugally, a prominent band of dark granules surrounded the equator of the egg. The position of this band was identical to the mitochondrial band previously delineated by the fluorochromes and vital dyes. Very often a clear indentation in the band indicated the presence of the maturation spindle.

The rest of the centrifugal zone consisted of lighter stained diffusely scattered granules. At the extreme centrifugal end of the egg, another empty, vacuolated area appeared overlaid by the prominent peripherally located black cortical granules.

The disposition of the mitochondria in the centrifuged egg was found to coincide with many of the granules that consistently fluoresced with lipophilic dyes (Richards, 1955; Metcalf and Patton, 1944). However, not all of the dyes showed that the mitochondria were concentrated in the equatorial zone of the centrifuged egg. This was the case after staining with rhodamin O (CI 45170), a vital dye and fluorochrome that has been used in the identification of mitochondria (Lillie, 1948).

Under U. V. illumination, eggs shed and dyed in a 0.001% solution of rhodamin in sea water revealed small, yellow orange particles evenly distributed throughout the cytoplasm. These same particles under dark field illumination appeared brilliant pink and had the additional advantage of being sharply differentiated from the natural luminescence of the cytoplasmic particles seen under dark field.

After staining, eggs were centrifuged at 33,000 *G* for 15 minutes in a pre-cooled head. The pinkish particles always formed a crescent-shaped band around the centripetal end of the germinal vesicle in the immature eggs.

In the mature eggs after GV breakdown, the pink particles were concentrated in a ring that was a part of the lipid cap in zone A. This ring formed a border around the centripetal edge of the cap (Fig. 22).

The appearance of the pink peripheral band around the lipid cap indicated that certain of the granules were distinctive from the general mass. This was also suggested from the results after Sudan black application to fixed centrifuged eggs which showed a thick peripheral band around the lipid cap. The specificity of rhodamin O for these particles strongly suggested that they represented a second type of mitochondria.

The ring was transitory in its appearance. A few minutes after the stratified eggs were removed from the cold, the pink particles of the band would move into the center of the lipid cap. Here they would form a diffuse oval of pink particles within the center of the silver white particles.

A similar centripetal band of granules lying around the periphery of the lipid cap was detected after fluorochroming with thioflavine.

Post-breakdown eggs were shed into a 0.0001% solution of thioflavine in sea water, stained for 10 minutes and centrifuged at 33,000 *G* for 15 minutes. When the preparations were excited with U. V. light (filters: 9863, 5970 or 18A) and observed with a noviol O filter, a definite yellow green band appeared centripetal to the blue violet appearing lipid cap. This band occurred in a position identical to that taken by the pink granules seen with rhodamin O.

An identical band of granules was demonstrated in living eggs that were dyed with toluidine blue. Eggs were freshly shed into 0.0001% solutions of toluidine blue (CI 52040) in sea water. Within ten minutes a heavy concentration of dark blue granules appeared around the germinal vesicle. This band graded off into smaller sparsely scattered blue granules throughout the rest of the cytoplasm. Around the periphery of the cell, just inside the cell membrane, a single row of dark blue granules was also found (Fig. 23).

After GV breakdown the blue granules were dispersed toward the periphery of the egg in the outer two-thirds of the cytoplasm. Upon prolonged staining in a dilute solution of the dye, smaller red violet metachromatic granules became apparent in the post-breakdown eggs. These were evenly distributed throughout the cytoplasm.

The dark blue granules were easily concentrated into the centrifugal hemisphere of the egg after centrifugation for 15 minutes at 4000 *G*. Here, the heavier yolk granules and the more centripetal zone of mitochondria all stained the same. The dark blue granules then graded off into the equatorial zone of the egg. The location of the metachromatic granules was clearly defined in the more heavily centrifuged eggs. Just centripetal to the clear yellow lipid cap in zone A, a ring of reddish purple metachromatic granules became concentrated (see Fig. 24). This sharply defined band was easily seen with natural light and appeared to be identical to the similar band of pink particles seen with rhodamin O under dark field illumination.

Since the metachromasia of these particles somewhat resembled that seen after eggs were stained with Nile blue sulphate, post-breakdown eggs were stained with each dye and centrifuged concurrently. The position of the metachromatic granules caused by toluidine blue was always quite distinct from those produced after application of Nile blue sulphate.

The hyaline region

Relatively few granules were seen in the hyaline region after centrifugation except those that drifted in from either of the adjacent stratified layers. The cytoplasm did exhibit a deep green fluorescence with acridine orange and acriflavine, thus suggesting a high nucleic acid concentration. Photographs of the centrifuged sea urchin egg taken with ultraviolet light showed the greatest absorption in the hyaline layer, thus indicating nucleic acid compounds in this layer (Harvey and Lavin, 1944). Similarly, the clear area stained pinkish blue with toluidine blue and pink with rhodamine B.

In the fixed egg, Nile blue sulphate colored the area light blue and Schiff's reagent in the plasmal test gave it a bright pink-violet color.

The presence of extremely small particles, rich in nucleic acid, in this hyaline area was indicated after the application of gallocyanin chrome-alum. The lower two-thirds of the hyaline zone contained a diffuse collection of dark blue particles.

The centrifugal pole region

This irregular hyaline area, just centrifugal to the yolk mass, constituted an enigma because of the apparent lack of particles present. With fluorescent dyes, it gave the same fluorescence as seen in the more centripetal hyaline band. None of the vital dyes indicated the presence of any granules.

However, in the Nile blue test on fixed eggs, this area appeared to be formed from a heap of irregular vesicles, each having dark blue granules around their borders. On the assumption that this area represented a structural phase of the cytoplasm, fixed centrifuged eggs were stained with gallocyanin chrome-alum at pH 0.8. The results indicated that a cone of very dark blue-black basophilic granules occupied the centrifugal pole, probably embedded in the structural phase of the cytoplasm.

Discussion and Conclusions

Each of the vital dyes and fluorochromes, when combined with centrifugation, provided specific information about one or more cellular inclusions in the oocytes of *Pectinaria*. From these results a composite picture of these inclusions in the centrifuged cell was projected, based upon their specific staining properties and stratification pattern.

In general, these investigations showed a stratification pattern as follows, moving from the centripetal to the centrifugal end of the egg: (1) oil droplets, (2) a cap of lipid granules, (3) a diffuse layer of fine granules, (4) a hyaline zone with the germinal vesicle (in immature eggs), (5) a broad layer of heavier granules, (6) a zone of heavy yolk spheres, (7) an irregular vacuolated area with scattered basophilic granules. Throughout the cortical region of the entire centrifuged egg were isolated clumps of cortical granules. A composite diagram of the centrifuged oocyte after germinal vesicle breakdown is shown in Figure 25.

The first inclusions at the centripetal pole were oil droplets that often fused into one or two large drops under prolonged centrifugation. Sudan black B turned them intensely black. These oil drops also stained bright red or pink when the Nile blue test was applied to the centrifuged fixed egg, which indicated the presence of neutral lipids.

The centripetal lipid granular cap reacted similarly. Sudan black formed a black cap, Nile blue sulphate turned it reddish violet in fixed eggs, and it stained an intense violet with Schiff's reagent in the plasmal test.

While the lipid cap could be easily seen under dark field or blue violet illumination, few of the fluorochromes used induced fluorescence in the lipid granules. An exception was thioflavine which induced yellow green fluorescence in the lipid cap.

The granular distribution found by Harvey (1941) in the sea urchin egg and in the annelid egg (1939) following centrifugation showed a mitochondrial band near the mid-region of the egg, just centripetal to the yolk mass. Monné (1944) and Monné and Hårde (1951) noted that centrifugation of the living sea urchin egg after staining with gentian violet or methylene blue resulted in the mitochondria concentrating at the extreme centrifugal end of the egg.

However, electron microscope studies of stratified sea urchin eggs by Lansing, Hillier and Rosenthal (1952) indicated that two layers of mitochondria stratified, one of high density just above the yolk granule layer and one of low density in the centripetal lipid layer.

In similar studies, Gross, Philpott and Nass (1956) reported that the mitochondria were concentrated in a layer centripetal to the yolk, yet they concluded that the mitochondria move to new positions both above and below the clear zone (zone B). Shaver (1957) also reported particles likened to mitochondria lying in an area just beneath the lipid cap (zone A).

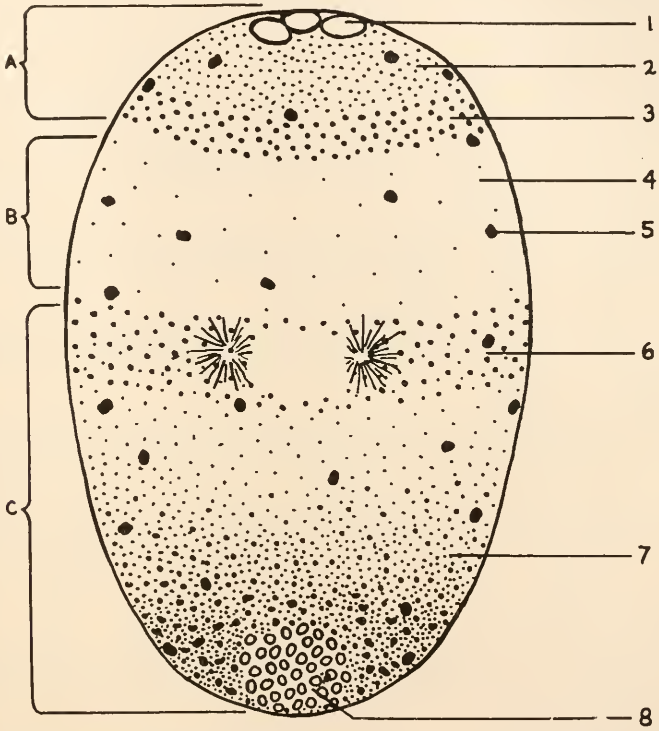

FIGURE 25. A composite diagram of the centrifuged oocyte of *Pectinaria* after GV breakdown. The stratified particles were stained by different vital dyes, fluorochromes and cytochemical tests. The three zones of the unstained centrifuged egg (A, B and C) are indicated, beginning with zone A at the centripetal pole. 1, Fat droplets. 2, Lipid granular cap. 3, Granular band I. 4, Hyaline zone with diffuse granules. 5, Cortical granules. 6, Granular band II, mitochondria and proteid yolk. 7, Dense proteid yolk. 8, Centrifugal vacuole with basophilic granules.

The relative distribution of particles identified as mitochondria in ascidian eggs seems to follow the same pattern. Reverberi (1956; 1957b) believes there are probably at least two kinds of mitochondria; one type is osmiophilic, does not stain with Janus green and localizes near the centripetal pole. The second type gathers above the equator and stains with Janus green. The position of the latter, however, conflicts with the finding of Mancuso (1959) and La Spina (1958) in eggs of *Phallusia*.

This description agrees with the evidence presented here of a second mitochondrial band existing just below the lipid cap in the centrifuged eggs of *Pectinaria*. The centripetal mitochondrial band is distinctive from the centrifugal band since it selectively stains bright pink with rhodamin O. Moreover, an identical yellow green fluorescent band centripetal to a blue lipid cap is also seen with thioflavine. Both of these acid fluorochromes are lipophilic. The lighter band of granules does not stain with Janus green B.

It should be noted, however, that a similarly transitory light blue band does form with crystal violet. Harvey (1941) found that the latter stained the centrifugal mitochondrial band purple (metachromatically) in the sea urchin egg.

A distinct band in the same position, just centripetal to the lipid cap and separated by a thin, clear band, was also detected following application of Schiff's reagent in the plasmal reaction.

More significantly, the centripetal band in the egg of *Pectinaria* reacted selectively with toluidine blue, forming a characteristic metachromatic reddish-violet band of granules just under the lipid cap.

Toluidine blue, a cationic dye, stains basophilic substances blue and reacts metachromatically with certain chromotropes as a result of polymerization of the basic dye (Michaelis, 1947).

The chemical interpretation of metachromasia is very tenuous without parallel confirmatory tests and controlled reactions. However, the substances that the metachromatic reaction detects are high molecular weight moieties with free anionic groups. This includes anionic mucopolysaccharides, both DNA and RNA nucleic acids and some anionic lipids capable of polymerization (Schubert and Hamerman, 1956). Some substances that cause metachromasia are heparin, chrondroitin sulphate and hyaluronate.

In a survey of metachromasia with toluidine blue, Kelley (1954) found metachromasia in eggs and ovarian tissue of many animal species, including *Arbacia, Chaetopterus* and *Spisula*. Metachromasia generally occurred in the cytoplasm and in the jelly around the eggs.

Metachromasia with toluidine blue was also noted by Dalcq (1957) in a study of the ascidian egg and in the developing rat egg (Dalcq, 1954) where he found a correlation between the localization of metachromatic granules and distribution of mucopolysaccharides.

Similar metachromasia occurs *in vivo* in the molluscan eggs of *Barnea* and *Gryphaea,* and in the sea urchins, *Psammechinus miliaris* (Pasteels and Mulnard, 1957) and *Paracentrotus lividus* (Pasteels, 1958). In these developing eggs stained with toluidine blue, they note very fine, blue granules (alpha granules) which are uniformly distributed throughout the cytoplasm. Upon centrifugation, these granules accumulate at the centrifugal pole. At the appearance of the sperm aster, new granules (beta granules) appear which are strongly metachromatic. The beta granules are believed to derive their metachromasia from the blue alpha granules when they become associated with the astral rays. With strong centrifugation, the beta granules are stratified beneath the lipid cap. From this and other evidence of acid phosphatase activity and acid mucopolysaccharides, Pasteels and Mulnard identify the beta granules as mitochondria.

These metachromatic granules are thus like those seen in *Pectinaria* in two respects, their reaction with toluidine blue and their position in the stratified egg. However, the centripetal band of metachromatic granules in *Pectinaria* is not seen associated with the astral rays.

Mulnard (1958) reported identical metachromatic α-granules and β-granules in the eggs of *Chaetopterus pergamentaceus* after they were stained with brilliant cresyl blue. He also described the presence of a third granule, material X, which he believed was the precursor of the β-granules.

The oocytes of *Spisula solidissima* were vitally dyed with methylene blue and toluidine blue by Rebhun (1959; 1960). Eggs left standing in dilute concentrations of either dye contained metachromatic granules which were extensively studied and analyzed. Rebhun found two sets of metachromatic particles, both directly stainable with toluidine blue. These particles measured ¼ to ½ micron in diameter initially and were uniformly distributed through the cytoplasm.

At the time of spindle formation, during the formation of the polar bodies or in the subsequent cleavages, Rebhun found that the metachromatic granules became associated with the aster. Both sets of granules migrated directly into the aster. This unusual behavior will be discussed in connection with Nile blue sulphate.

When the oocytes were centrifuged before GV breakdown (8000 *G* for 1½ to 4 minutes), the granules were mainly located in a narrow layer centrifugal to the germinal vesicle. A few were found in the lipid cap and in the centrifugal yolk area.

After GV breakdown, centrifugation stratified the metachromatic granules in two locations, a layer at the centripetal end of the hyaline zone and a layer at the centrifugal end of the yolk area.

Rebhun equates the centripetal particles with the β-granules of Pasteels (1958) on the basis of their location in the centrifuged egg and their migration into the asters. He also identifies the centrifugal particles with the α-granules of Pasteels *et al.* but these are not astrally located according to Pasteels and Mulnard (1957).

On the basis of their metachromatic staining and stratification in the centrifuged egg, the centripetal granules in the oocyte of *Pectinaria* appear to be very similar to the centripetal layer of metachromatic granules that Rebhun finds in *Spisula*. However, the failure to see the metachromatic granules in *Pectinaria* associated with the asters during the first polar body formation cannot be explained from the present findings. It is possible that they were not detected or that they do not appear until after fertilization.

Demonstration of the proteid yolk particles in the living egg was easily accomplished even though the dyes were not always specific. Proteid yolk fluorescence was orange with acridine orange, yellow green with thioflavine and yellow with acriflavine. These granules were readily vitally stained pink with neutral red, deep blue with toluidine blue or Nile blue sulphate.

The reaction of similar dyes in the sea urchin led Monné (1944) to suggest that the yolk is composed of a phosphoprotein combined with lipids. Later, differential staining of various polysaccharides caused Monné and Slautterback (1950) to conclude that the yolk probably consisted of aminopolysaccharide combined with a protein and lipid.

Raven (1958) subdivides the yolk granules of *Limnaea* into fatty yolk, consisting of free lipids and fat globules, and proteid yolk. The latter, composed of two granular types, is rich in mucopolysaccharides.

The specificity of auramine for the proteid yolk granules in the eggs of *Pectinaria* indicates the presence of mucopolysaccharides, probably in loose combination with proteins.

Just centrifugal to the hyaline layer in the pre-and post-GV-breakdown egg of *Pectinaria*, an equatorial group of granules is concentrated into a band just centripetal to and overlapping with the yolk granules. The identification of a similar band in centrifuged eggs of the sea urchin (Harvey, 1939, 1941, 1944) and in the eggs of *Chaetopterus* (1939) and *Nereis* (Costello, 1939, 1958) strongly suggests that the band is rich in mitochondria.

Differential fluorescence of this granular band was produced with thioflavine, acridine orange, acriflavine and phosphine. Crystal violet also selectively stained it. Less specific but positive identification was obtained with Janus green and Sudan black B.

In the sea urchin egg Harvey (1941) was able to stain the mitochondria with both gentian violet and Janus green. Monné (1944) also reported mitochondrial staining with gentian violet.

The specific staining of similar stratified granules in *Pectinaria* by Janus green and gentian violet suggests the presence of mitochondria in this band.

Induced fluorescence of granules in the same stratified position by acridine orange, acriflavine, thioflavine and phosphine suggests that these fluorochromes are also staining the mitochondria. In particular, thioflavine, which is lipophilic, also induces fluorescence in the mitochondrial band just centrifugal to the lipid cap.

The possibility still exists that the induced fluorescence is caused by similarly localized but not identical granules since the mitochondria do overlap with the yolk granules. In the centrifuged eggs of *Limnaea stagnalis,* Raven (1958) also finds a mixture of mitochondria and γ-granules, one type of proteid yolk, in an analogous position. While the fluorescence of this band of granules with the above fluorochromes under the described conditions appears to be specific, the exact nature of the granules fluorescing requires further verification.

In the living eggs of *Pectinaria* Nile blue sulphate produced striking metachromasia of granules that localized in a band corresponding to the position of the mitochondria. This was also confirmed in whole fixed centrifuged eggs. The metachromasia produced indicated the granules contained glycolipids.

In *Pectinaria* the astral granules also have their origin in the metachromatic granules produced by Nile blue sulphate. It would be tempting to assume that the metachromatic astral granules were mitochondria. Gustafson and Lenicque (1952) used Nile blue sulphate to follow the mitochondria in the developing sea urchin egg. They postulated that the stain adhered to the lipid-rich sheath that surrounds the mitochondrium. However, they did not report that the granules were metachromatic.

Raven (1958) indicated that the mitochondria in *Limnaea stagnalis* gather around the maturation spindle and often migrated in between the astral rays. He also noted that the aster was surrounded by the centripetal γ-granules that were distinct from the centrifugal proteid yolk granules.

Except for their stratified position in the mitochondrial layer, there is not too much evidence to indicate that the metachromatic astral granules in *Pectinaria* are mitochondria. They also differ from the granules stained with mitochondrial dyes and fluorochromes in that the astral granules are only seen with Nile blue sulphate.

In addition to the metachromatic astral granules in *Pectinaria,* similar granules have been seen in related eggs by others.

Taylor (1931) described natural red granules dispersed through the cytoplasm of the echiuroid, *Urechis caupo.* In the centrifuged egg they gathered in clusters at the extreme centrifugal end of the egg. When the amphiaster formed during maturation and cleavage, in the normal or centrifuged egg, the granules migrated along the astral rays and finally surrounded the nucleus of each daughter cell.

Iida (1942) also followed particles stained with neutral red along the astral rays and along the mitotic spindle in the sea urchin egg. These granules were also distributed to the daughter cells.

In this connection, Lillie (1906) observed that neutral-red-stained granules collected in a ring around the first maturation spindle in *Chaetopterus* oocytes. He indicated that they were derived from granules composing the "residual substance" or the germinal vesicle.

It is fairly certain that this is not the case in oocytes of *Pectinaria.* However, a thin layer of granules, perhaps equivalent to the residual substance, does stratify *within* the germinal vesicle. It is possible that the latter granules are those which layer just centrifugal to the lipid cap after GV breakdown in *Pectinaria* (see Fig. 26).

Vitally stained granules were observed by Kojima (1959a, 1959b) in several sea urchin eggs and in the egg of *Urechis unicinctus.* In the fertilized eggs that were stained with neutral red, toluidine blue, Janus green or Nile blue sulphate, deeply stained granules appeared around the aster. In the unfertilized egg the granules collected around the germinal vesicle. After strong centrifugation, the granules displaced into the centrifugal pole of the egg.

It was difficult to equate the metachromatic astral granules of *Pectinaria* with these varied findings. First, the nature of the granules was not clearly established. The neutral-red-staining granules seen by Lillie were obviously different. Those reported by Iida and Kojima were not observed in *Pectinaria.* The data of Kojima for Nile blue sulphate and toluidine blue indicated different levels of stratification and lack of metachromasia.

In *Spisula* oocytes Rebhun (1959) found two types of metachromatic particles, the centripetal β-particles already referred to and the α-particles which layered in the centrifugal hemisphere. Both types migrated into the asters and each was excluded as being mitochondria.

It is not likely that the metachromatic granules produced by Nile blue sulphate in *Pectinaria* can be identified with the α-particles seen with toluidine blue in *Spisula.* First, the two dyes in question stain different particles in the eggs of *Pectinaria.* Secondly, the two sets of particles stratify in different layers upon centrifugation, the astral granules being derived from a layer that is coincident with the mitochondria in *Pectinaria*. This is not the case in *Spisula*.

In electron microscope studies, Rebhun (1960) identified the metachromatic granules as multivesicular bodies and concluded that the metachromatic granules were definitely not lipid, mitochondrial, yolk or cortical granules. Two other components were present, the Golgi bodies, particularly plentiful in early oocytes, and annulate lamellae which occasionally orientated with the asters. It is possible that one of these particles is analogous to the astral granules of *Pectinaria*.

The nucleolus of the mature oocyte of *Pectinaria,* characteristically an amphinucleolus, was strongly basophilic in the cortical region but the nucleolar vacuoles remained unstained (Fig. 26). All of the vital dyes and fluorochromes applied to the oocytes indicated that the body of the amphinucleolus was principally composed of DNA. This was confirmed after treatment of the fixed eggs with the Feulgen reagent. When the eggs were previously extracted with 4% trichloracetic acid at 90° for 15 minutes, the nucleolus remained unstained following the Feulgen test.

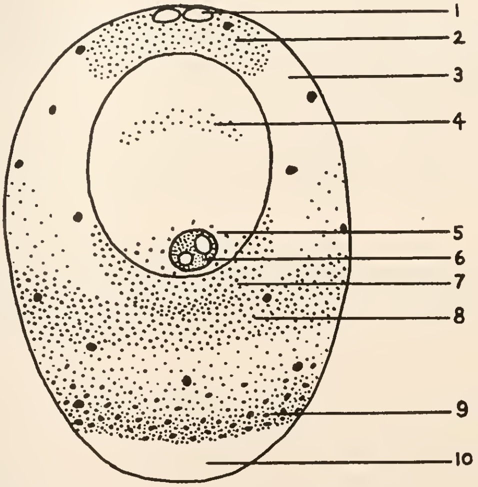

FIGURE 26. A centrifuged immature oocyte, showing particle distribution in relation to the intact germinal vesicle. 1, Fat droplets. 2, Lipid granular cap. 3, Hyaline zone. 4, Germinal vesicle substance. 5, Basophilia. 6, Amphinucleolus. 7, Granular Band I. 8, Granular Band II, mitochondria and proteid yolk. 9, Dense proteid yolk. 10, Centrifugal vacuole.

Kobayashi (1953, 1954) observed similar amphinucleoli in the eggs of the oyster (*Ostrea laperousi*) which were Feulgen-positive. He also found that the karyosome stained with methyl green and the plasmosome with pyronin, indicating the presence of RNA.

Another type of amphinucleolus was reported by Sawada and Murakami (1959) in *Mactra veneriformis*. In this form, nucleolus 1 stained deeply with pyronin while nucleolus 2 gave a weak reaction with pyronin.

Attempts to demonstrate RNA in the non-staining intranucleolar buds of the mature oocytes in *Pectinaria* were unsuccessful, while in very young oocytes both epinucleolar and intranucleolar buds fluoresced bright red with acridine orange and stained metachromatically with thionin. Acridine orange stained RNA nucleoli in tumor cells bright red (Tweedell, 1960a).

Changes in the amphinucleoli during the growth phase are well known in molluscan eggs; Raven (1958) and Wilson (1925) report on numerous cases of variation in the amphinucleoli of annelids, molluscs and arthropods. This appears to be the case in the nucleoli of the developing oocytes of *Pectinaria*.

Summary

1. Living eggs of *Pectinaria gouldii* were stained with vital dyes and vital fluorochromes before and after germinal vesicle breakdown. Observations were made with the bright field, dark field and fluorescence microscopes. Changes in the germinal vesicle, nucleolus and chromosomes of the living eggs were followed.

2. Other eggs were vitally dyed and centrifuged in order to stratify the cell particulates. Cell granules were identified, based upon their specific staining and their stratified position in the centrifuged egg. These included lipid droplets, lipid granules, mitochondria, proteid yolk, basophilic and cortical granules. Limited cytochemical tests were made to verify their identity.

3. Two kinds of metachromatic granules were seen. With toluidine blue, the granules occur at the centripetal pole just beneath the lipid cap. When Nile blue sulphate was applied, a different metachromatic band stratified just centripetal to the heavier yolk granules. Astral granules originate from the latter metachromatic band and became associated with the first maturation spindle.

4. The fluorescent cell components included cell and nuclear membranes, nucleoli, chromosomes, lipid granules, two types of yolk granules and mitochondria.

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OXYGEN UPTAKE IN SHORT PIECES OF TUBULARIA STEMS

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Short pieces (1–1½ mm. long) of *Tubularia* stems reconstitute miniature hydranths or partial hydranths. Unusually long periods are required for reconstitution of these pieces, and hydrogen ion estimates with indicators show low pH of the coelenteric fluid of such pieces (Miller and Miller, unpublished data). Since increased O_2 in the surrounding sea water increases the number of pieces which reconstitute and the size of the organ primordia, it became of significance to determine whether or not the oxygen uptake of 1- and 1½-mm. pieces was depressed as compared with that of longer stem segments.

In addition, the previously published studies on oxygen uptake in *Tubularia* (Hyman, 1926; Barth, 1940b; Sze, 1953) have resulted in a certain degree of confusion regarding the role of oxygen in reconstitution. On the one hand, whether or not a hydranth develops and the size of the hydranth which reconstitutes at a cut surface depend upon the oxygen available to the stem (Barth, 1938; Miller, 1937, 1942; Zwilling, 1939); on the other hand, oxygen uptake measurements during reconstitution failed to show that reconstituting stems used an appreciable amount of oxygen more than stems with both ends ligatured (Barth, 1940b). Since reconstitution in *Tubularia* involves cell migrations (Bickford, 1894; Steinberg, 1955), dedifferentiation and redifferentiation (Bickford, 1894), all processes which require energy, logic would demand an appreciable increase in O_2 uptake during these activities. Likewise, there were certain technical problems in both Barth's and Sze's methods which made it desirable for their results to be checked in another laboratory. Therefore, a new study of oxygen requirements in *Tubularia* was initiated in the summer of 1961 using the cartesian diver equipment of the Single Cell Research Foundation.[1]

MATERIALS AND METHODS

Specimens of *Tubularia crocea* were collected in the region of Woods Hole, Mass., and were maintained in aerated running sea water in the laboratory. During the first half of the summer they came from the warmer waters of the south side of Cape Cod. During the last half they were collected from the Cape Cod Canal where the temperature seldom rises above 15° C.

Straight stems of uniform thickness were selected from a single bunch of the stock supply and placed in filtered sea water to which had been added 16×10^{-5} gm./ml. streptomycin to provide bacteriostasis. The stems remained in this solution in a cold room (18° C.) for at least 12 hours. The hydranth plus 5 mm. of the stem were then removed and the required length of stem was cut from the

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[1] Reported by abstract: *Biol. Bull.*, 121: 398, 1961.

region immediately proximal. Since the accuracy of the results depended upon the accuracy of the measurements of length and diameter of the stem segments, the sizes of the pieces were checked under a microscope with an ocular micrometer, and stems which varied were discarded.

Oxygen uptake measurements were made in the Claff modification of the Holter (1943) and Linderstrøm-Lang (1943) cartesian diver apparatus with 7 flotation tubes suspended in a water bath maintained at 18° C. ±.01° C. For each series of measurements six of the tubes contained divers with tubularian stems and the seventh contained an unfilled diver to act as a check on the equipment. A "braking" pipette (Claff, 1947) was used to insert stem pieces plus 2 mm.^{3} of incubating solution into the divers. The necks of the divers were sealed with 2 mm.^{3} NaOH to absorb CO_2 and 2 mm.^{3} paraffin oil to prevent diffusion of gases. After sufficient time for the divers to equilibrate, manometer readings were taken every 10 minutes for a minimum of two hours.

RESULTS

Since oxygen uptake data obtained on single 3-mm. stems were used as a standard of reference, in each set of measurements at least one diver contained a 3-mm. stem, the "control." In the various experiments to be described the oxygen up-

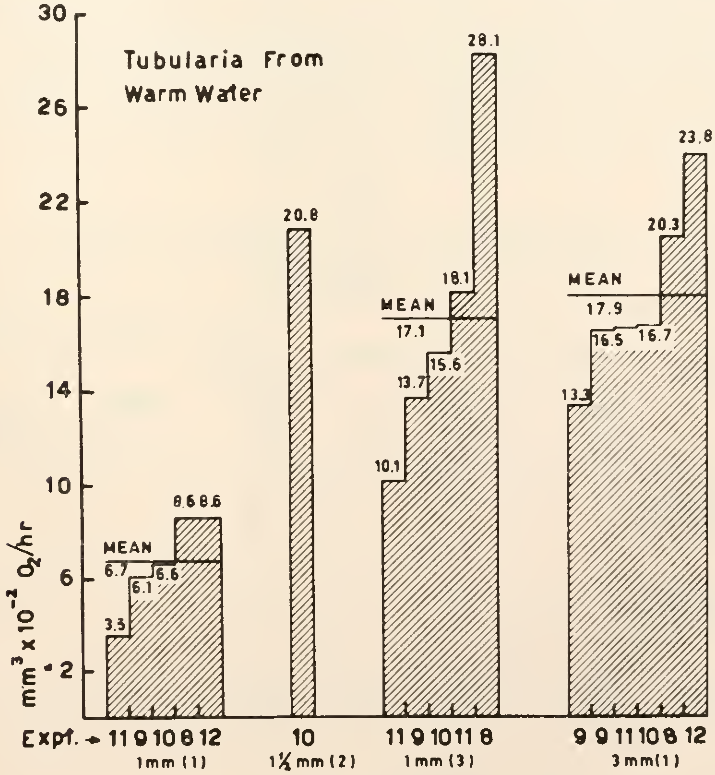

Figure 1.

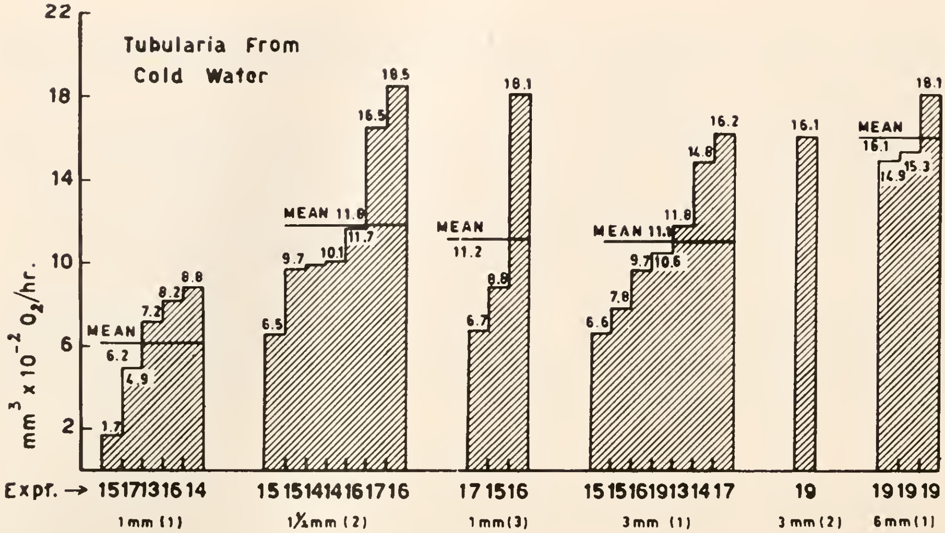

FIGURE 2.

takes of the following were compared with that of a single 3-mm. stem: (1) a single 1-mm. piece, (2) three 1-mm. pieces, (3) two 1½-mm. pieces, (4) two 3-mm. pieces and (4) one 6-mm. piece (Figs. 1, 2).

Table I summarizes the O_2 uptake measurements on 43 stems of various lengths from 1 mm. to 6 mm. and measured either singly (Columns 1, 4, 6), in pairs

TABLE I

O_2 UPTAKE OF 1, 1½, 3 AND 6 MM STEMS OF TUBULARIA

Source of Stems	Total Stem Length 3 mm			Total 1 mm	Total Length 6 mm	
	3 mm ▭	1½ mm ▭	1 mm ▭	1 mm ▭	3 mm ▭	6 mm ▭
Warm Water (WOODS HOLE)	17.9	20.8*	17.1	6.7		
Cold Water (C.COD CANAL)	11.1	11.8	11.2	6.2	16.1*	16.1
MEANS	14.2	13.0	14.9	6.4	16.1*	16.1
Column	1	2	3	4	5	6

* Tentative: More data needed

(Columns 2, 5), or in threes (Column 3). As may be seen in the averages little difference was found between the oxygen uptake of one 3-mm. stem (Column 1), two 1½-mm. stems (Column 2) or three 1-mm. stems (Column 3) in the same diver. However, when only one 1-mm. stem was placed in a diver (Column 4), it used nearly half as much as three 1-mm. stems. Similarly, although two 3-mm. stems (Column 5) used the same amount of O_2 as one 6-mm. stem (Column 6), a single 3-mm. stem used $\frac{2}{3}$ as much as two 3-mm. stems or one 6-mm. stem.

Table I also shows that the stems collected early in the summer (from Woods Hole) had appreciably higher O_2 uptake than those collected from Cape Cod Canal, although the general relationships between the effects of size of the piece and crowding appeared to be similar. In order to assess the validity of these impressions the data in each series were calculated as per cent of the O_2 uptake of the 3-mm. stem which served as "control" for that series, and these are summarized in the next table.

TABLE II

UPTAKE IN PERCENT OF OXYGEN USED BY SINGLE 3MM STEM

Source of Stems	Total Length 3mm			Total Length	Total Length 6mm	
	3 mm	1½ mm	1 mm	1 mm	3mm	6mm
	▭	▭▭	▭▭▭	▭	▭	▭
Warm Water (WOODS HOLE)	100%	125%*	91 %	36%		
Cold Water (C.COD CANAL)	100%	110 %	116 %	51 %	152%*	152%
MEANS	100%	112 %	100%	44%	152%*	152%

* Tentative: More data needed

In Table II it is seen that two 1½-mm. stems used 12% more O_2 than one 3-mm., and that three 1-mm. stems used the same amount as the one 3-mm. stem. One 1-mm. stem used 44% of that used by one 3-mm. and two 3-mm. stems or one 6-mm. stem used 152% of that of the 3-mm. control.

Since the data for stems from cold water are more complete than those for stems from warm water, the former were used for a further analysis of the situation. If the cut surface were the determining factor in O_2 uptake, the 1-mm., 3-mm., and 6-mm. pieces should have the same uptake since they all have two cut surfaces; instead, the actual measurements give a 1:2:3 ratio. Likewise, two 1½-mm. pieces should use two times as much O_2 as one 3-mm. piece and three 1-mm. pieces should require three times that of one 1-mm. piece. The actual findings were 10% and 61% increases, respectively.

If O_2 uptake were equal along the entire length of stem, all of the 3-mm. combinations should give the same uptake. The observed findings of 110% for two 1½-mm. pieces and 116% for three 1-mm. pieces could perhaps be reconciled to this hypothesis; however, the observed uptake of the single 1-mm. stem is too high (51% instead of 33% of the 3-mm. uptake) and that of two 3-mm. stems or one 6-mm. stem is too low (152% of the control uptake rather than 200%).

Table III shows the precise relationship between O_2 uptake and length of stem when calculated per cut surface. In the upper row it is seen that each cut surface of a 6-mm. stem was associated with an uptake of oxygen which was 1½ times that of a 3-mm. stem and 2½ times that of a 1-mm. stem. In the second row it is seen that when there were four cut surfaces for 6 mm. of stem (*i.e.*, two 3-mm. pieces) the uptake per surface was reduced, as was the case with a 3-mm. piece cut into two 1½-mm. pieces. However, oxygen exchange per cut surface again was greater in the longer than the shorter pieces. That this was the result of a true inhibition of oxygen consumption was shown when the uptake per cut surface of two 3-mm. pieces in a diver was compared with that of a single 3-mm. piece (4 as compared with 5.3 $mm.^3 \times 10^{-2}$ per hour) and that of two 1½-mm. pieces or three 1-mm. pieces compared with that of a single 1-mm. piece in a diver (3.0 or 1.9 as compared with 3.1).

TABLE III

OXYGEN-UPTAKE PER CUT SURFACE*

No. of Cut Surfaces	Length of Stem		
	6 mm	3 mm	1 mm
2	8.1	5.6	3.1
4	4.0	3.0	-
6	-	1.9	-

* $mm^3 \times 10^{-2}$ per hour

The influence of total volume of tissue upon oxygen uptake is shown in Table IV, in which the data on the stems from cold water have been calculated on the basis of uptake of oxygen per millimeter of stem length. These show that when the distance between the two cut surfaces is great the average uptake is small, when the distance is small the average uptake is large. The data are not sufficient to quantitate the differences in oxygen requirements of the 1–1½ mm. at the two ends of the stem which have been activated by exposure to oxygen and the intervening non-activated stem. However, the difference between the uptake of single 6-mm. stems and single 3-mm. stems (16.1 minus 11.1 mm.^{3} × 10^{-2}) suggests that under the conditions of the experiment the non-reconstituting parts of the stem consume oxygen at the rate of something in the order of 2 mm.^{3} × 10^{-2} per millimeter length as compared with 5½ for the ends. Further studies are planned in order to verify this finding.

The lower half of Table IV again shows the inhibitory effects of increasing the number of cut surfaces per mass of tissue when confined in a small volume of fluid. Because oxygen uptake depends on oxygen concentration of the medium (Barth, 1938), calculations were made to determine the volume of oxygen in the divers at the beginning of the period of measurement. Since the mean volume of the divers was 68.85 mm.^{3}, they contained approximately 13.77 mm.^{3} of O_2. Even using a rate of O_2 uptake of 30 mm.^{3} × 10^{-2}/hr. (higher than any which has been measured), the oxygen in the diver would suffice for 46 hours. Therefore, hypoxia could not have contributed to the reduction in uptake.

TABLE IV

OXYGEN UPTAKE PER MILLIMETER LENGTH OF STEM*

No. of Cut Surfaces	Length of Stem 6mm	3mm	1mm
2	2.7	3.7	6.2
4	2.7	4.7	-
6	-	4.5	-

* mm^3 x 10^{-2} per hour

Since CO_2 is a potent inhibitor of reconstitution, calculations were made to determine whether the volume of NaOH solution in the necks of the divers was adequate to absorb the CO_2 liberated. Using the same figure of 30 mm.^{3} × 10^{-2}/hr.

O_2 uptake the calculations showed that the 2 ml. of NaOH could absorb CO_2 for 75 hours before becoming exhausted. This indicates that some inhibitor other than CO_2 liberated by the cut ends of the stems was responsible for the O_2 depression. Since it has been demonstrated that low pH inhibits reconstitution (Goldin, 1942) and that pH-lowering substances are released during reconstitution (Miller, 1948; Miller and Miller, unpublished data), it is suggested that these substances may depress the O_2 uptake in the divers containing two or more pieces.

DISCUSSION

1. Oxygen uptake and reconstitution

Barth (1940b) was unable to find any differences in O_2 uptake of "regenerating" stems (with open ends) and "non-regenerating" stems (with both ends ligatured) and concluded that very little oxygen was used in regeneration, even though his earlier studies had shown that the process was highly oxygen-dependent. However, there are aspects of his technique that make his findings difficult to interpret. His determinations were made in a Warburg apparatus which was shaken during the measurements. This so greatly increased the oxygen available to the stems that ligatured stems can hardly be considered as resting stems. Indeed, he reported (p. 372) that 50% of one group of ligatured controls formed hydranths. Under ordinary circumstances ligatured stems do not show any visible signs of reconstituting. Therefore, it is possible that the lack of difference in oxygen uptake between ligatured and non-ligatured stems could be attributed to an artificially elevated uptake in the ligatured stems, caused by the shaking in the Warburg apparatus. In spite of this possibility Barth's conclusions regarding oxygen uptake in reconstitution have been widely quoted and have been incorporated in theories of regeneration (Barth, 1940a, 1944; Spiegelman, 1945; Steinberg, 1954, 1955).

In 1953 Sze reported a study of oxygen uptake in *Tubularia* stems using cartesian divers. His technique avoided the problems raised by shaking but encountered other problems which again complicate the interpretation of the results. He found it necessary to make his uptake measurements at a temperature of 25° C. even though the stems had come originally from colder water and had been kept in the laboratory at 15° C. *Tubularia* colonies from colder water that are brought into a laboratory at 25° C. lose their hydranths and may even cytolyze (Moore, 1939). Stem segments are less sensitive than hydranths, but can hardly be considered as normal under such conditions of temperature stress.

In addition, both Barth and Sze reported their measurements in $mm.^3/hr./10$ mg. dry (or in some cases wet) weight. Although theoretically this should be the most precise procedure, in the case of *Tubularia* the presence of the chitinous perisarc introduces a complication which negates its advantages. Since the nonliving perisarc far outweighs the metabolizing tissues of stem, calculations based on weight will contain a large error if there are differences in thickness in different parts of the perisarc. Such differences are slight and probably can be safely disregarded in short pieces from adjacent regions. However, the thickness of the perisarc increases proximad and the differences become appreciable in sections only a few millimeters apart. To avoid this complication, in the present study the lengths and diameters of the stems were measured under magnification and O_2 uptake comparisons were made on the basis of units of stem length.

Our data on the oxygen uptake of pieces less than 3 mm. long do not offer much assistance in resolving the question of whether or not reconstitution is accompanied by an appreciable alteration in oxygen uptake. However, ciliary activity, production of pH-lowering substances and the subsequent differentiation of a hydranth all indicate that under ordinary conditions, from 1 to 1½ mm. of stem subjacent to the cut surface is involved in the activation which follows sectioning. On this basis, the difference in uptake between a 3-mm. and a 6-mm. stem was used to compare the uptake of the peripheral 3 mm. with the interior 3 mm. This showed that the average oxygen uptake per millimeter of stem at the ends of the stem was two times that at the middle (10.6/3 or 3.5, as compared with 5.5/3 or 1.8). We have evidence (unpublished) that there is balance during reconstitution between the level of oxygen available to the cells and the level of pH-lowering substances which accumulate in the stem and which are inhibitory (Goldin, 1942). Because of coelenteric circulation, the concentration of these inhibitors is lower in long than in very short stems (Miller, 1948; Miller and Miller, unpublished data). Therefore, it is entirely possible that when longer stems are measured, the uptake of the reconstituting ends will be found to be appreciably greater than two times that of the resting stem tissue. However, the important fact remains that the measurements reported here bring the changes in oxygen uptake following cutting into a rational relationship to the well known dependence of reconstitution upon oxygen which Barth demonstrated so clearly in 1938. The measurements reported here also accord with studies on the energy requirements in *Corymorpha*, a related species which has a naked stem (Child and Watanabe, 1935), in hydranth development in *Tubularia* embryos (Miller, 1946) and in embryological processes and regenerative phenomena in general (Child, 1941).

2. *Oxygen uptake in 1-mm. pieces*

Very short pieces present an interesting complication. Since their length is less than that of a normal reconstituting hydranth and they have two surfaces for metabolic exchange, one might expect unusually large hydranth primordia in these short pieces. Such is not the observed result. They often fail to reconstitute at all and when reconstitution does occur, they produce the smallest hydranth primordia or fully formed hydranths that the authors have seen. Other evidence of inhibition in these short pieces is that instead of completing reconstitution in 48 to 60 hours they often require 4 to 5 days.

In spite of this, the single 1-mm. pieces gave the highest per millimeter O_2 uptakes of any of the pieces measured. In a parallel study (Miller and Miller, unpublished data) it has been found that the 1-mm. stems have the lowest average pH of any stems studied. Thus it appears that the antagonism between acid and O_2 reported by Goldin (1942) has a counterpart in reverse within the coenosarc of very short (1-mm.) stems. In spite of increased availability of O_2 for the tissues and increased utilization by them, in the presence of increased acidity reconstitution is delayed, and when it occurs is inhibited (*i.e.*, the scale of organization is reduced). If this picture is a correct one, increasing the O_2 in the sea water should increase the scale of organization (*i.e.*, the size of the organ primordia). When tested, this prediction was verified. Oxygenation so increased the size of the primordia that the pieces were not long enough to produce complete hydranths. As a result there was a great increase in the number of partial forms possessing a hypostome, distal tentacles and gonophore buds or merely a hypostome and distal tentacles. Measurements showed that 10 times as much tissue was included in the distal tentacles of the latter group as in the distal tentacles of the complete hydranths which developed in the unoxygenated controls (Miller and Miller, unpublished).

3. Inhibition of reconstitution

Reconstitution in *Tubularia* is initiated by the oxygen which enters the stem through the cut surfaces. However, this form is extremely sensitive, and reduction in size or total inhibition of the developing hydranth can be produced by a wide variety of agents of both exogenous and endogenous origin. The present discussion will be limited to naturally occurring inhibitors. In 1939 CO_2 was reported to be a powerful inhibitor (Miller, 1939) and later it was shown that effect was produced by hydrogen ions (Goldin, 1942). Subsequently it was found that pH-lowering substances accumulate in the coelenteron of reconstituting stems and especially in the reconstituting hydranth. The concentration in ligatured stems reaches levels which Goldin found to be inhibitory when externally applied (Miller, 1948; Miller and Miller, unpublished data). At one time Barth (1940) postulated competition for nutritive substances circulating within the coelenteron to explain dominance of the distal over the proximal cut surface. However, his data could be interpreted equally well on the hypothesis that dominance was maintained by a differential susceptibility to inhibitors (Child, 1941). When put to a test, the stems through which fresh filtered sea water flowed throughout the period of regeneration actually produced slightly more hydranths than did controls from which no coelenteric fluid was removed (Miller, 1959). In this experiment any nutritive substances liberated into the coelenteron of the experimental stems were removed before they could reach the distal end, since the flow was from distal to proximal.

Rose and Rose (1941), Rose (1955), Tardent (1955, 1960) and Tweedell (1958) have been interested in inhibitors produced by hydranths and stems. Although Fulton (1959) reported that he obtained inhibition from hydranth water only when he could demonstrate bacterial multiplication in the preparations, he found that preparations either from hydranths or stems made by extraction (Tardent, 1955; Tweedell, 1958) contained inhibitors which were not dependent upon bacterial action (Fulton, 1959, p. 237). Likewise, Rose (1957) reported polarized inhibitory effects in grafting experiments which could not be explained on the basis of contaminants. Also, Beloussov and Geleg (1960) have reported inhibition which was independent of bacterial action.

Although many authors have reported inhibition of regeneration in *Tubularia* resulting from crowding, the present observations are the first which show that under these conditions the oxygen uptake is depressed. Calculations showed that because of the relatively large volume of the air and small volume of sea water in the divers, no oxygen deficiency could develop in the period of the measurements. Likewise, the NaOH in the divers was found to be more than adequate. Thus it was concluded that some other noxious product of metabolism was involved primarily in this effect.

During the first twelve hours of reconstitution the ends of the stems liberate substances into the coelenteron which increase the hydrogen ion concentration at the ends by a factor of 12 (1.2 pH units) and maintain it at this level throughout the remainder of the reconstitutive period (Miller, 1948; Miller and Miller, unpublished data). This indicates a high rate of production of acidifying substances. As shown by Goldin (1942) a pH of 6.8 in the surrounding sea water will prevent hydranth formation at ordinary levels of oxygenation (5 cc./l.). It is suggested that in the small volume of sea water in the divers, pH may have fallen rather rapidly to inhibitory levels.

It must be emphasized, however, that these studies were made during the first 6–8 hours after cutting. They give information only during the migratory phase of reconstitution. Other and organ-specific inhibitors, such as those indicated in Rose's work (1957), undoubtedly operate during later stages. They may likewise affect oxygen uptake but as yet there is no information on this question.

SUMMARY

Oxygen uptake measurements were made in cartesian divers on 43 pieces of *Tubularia* stems between 1 mm. and 6 mm. in length with the following findings:

1. The 1-mm. stems had the highest, 3-mm. stems the next highest and 6-mm. stems had the lowest uptake when calculated per millimeter of length of stem.

2. By comparing uptake of 3-mm. and 6-mm. stems it was found that the middle 3 mm. of the 6-mm. stems used O_2 at less than half the rate of the two ends. This is in disagreement with the conclusions of Barth and Sze that regeneration does not involve any appreciable increase in oxygen requirements.

3. When two or more pieces were placed in the same diver their oxygen uptakes were depressed. Calculations showed that neither hypoxia nor hypercapnia could have caused this depression. It was suggested from other studies that acid metabolites liberated through the cut surface may have caused the observed effects.

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ABSTRACTS OF PAPERS PRESENTED AT THE MARINE BIOLOGICAL LABORATORY

1962

ABSTRACTS OF SEMINAR PAPERS

July 3, 1962

Amino acid transport in the human erythrocyte: kinetics and mechanism. Peter Rieser.

The penetration of the human erythrocyte surface by L-valine was studied densimetrically. "Exit" experiments were done with cells preloaded with 100, 75, and 50 millimolar solutions of the amino acid. The rates of transfer varied inversely with the differences in valine concentration across the membrane. The results show that valine does not penetrate the cell via Fickian diffusion. Instead, the same data fit the near-saturation carrier equation with an experimentally determined value of 4 millimoles for the half-saturation constant. Cells exposed to a variety of endopeptidases and lipolytic enzymes failed to exhibit altered penetration rates for amino acids (valine, leucine). Cells exposed first to trypsin and then to lipose became completely impermeable to amino acids but retained an intact glucose transport system. This suggests a binding site of lipid nature with which amino acids temporarily combine in being transferred across the cell surface.

Some effects of ionizing radiations on the embryo. Roberts Rugh.

The response of the embryo to ionizing radiations indicates that at all stages it is more radiosensitive than is the adult into which it develops; its cells cannot recover from irradiation insult so that they are either eliminated (and phagocytized), resulting in a deficit embryo, or remain as abnormal cells to interfere with development and cause congenital anomalies; and, in contrast with the adult, the embryo has unique powers of realignment of its cells which have not been differentiated so that a topographically well-balanced organism may result. Cell deficiencies may be expressed as stunting, microphthalmia, microcephaly, or the actual loss of an organ. Anomalies appear largely to affect the central nervous system and the sense organs, probably because there are so many neuroblasts at all times following the initiation of differentiation and until after birth. The primitive neurectoderm is relatively radioresistant (400 r), and the neuron is very radioresistant (10,000 r) but the neuroblast (as with any -blast stage for any tissue) is highly radiosensitive, being killed by 25 r. On the basis of examination of over 20,000 mouse embryos and fetuses, it may be wise to consider establishing a limit of 10 r to the human embryo during the first 6 weeks following conception.

Electron microscopy of neurosecretory cells in the preoptic nucleus of the toadfish (Opsanus tau). Ernst Scharrer.

The neurosecretory cells of the nucleus praeopticus of the toadfish (*Opsanus tau*) appear to contain only one kind of granule when examined with the light microscope. However, in electron micrographs vesicles and granules showing marked differences in size and density are seen in the perinuclear zone in much the same manner as in the goldfish (Palay, 1960). The neurosecretory granules of the toadfish are identifiable by their size (±3000 Å) and high electron density. They are formed by the Golgi apparatus. In addition, there are large (±1 μ) round vesicles whose content in Epon-embedded material appears finely granular and much less electron-dense than that of the neurosecretory granules. These large inclusions are frequently open on one side, and their content mixes with the cytoplasmic matrix. The rupture of the membrane may occur during the time it takes for the fixing fluid to reach and penetrate the cell. These and other unclassifiable constituents of similar size with various lamellar and vesicular inclusions are distributed throughout the perinuclear zone. Such bodies are not characteristic of secreting neurons and may be found also in non-nervous cells.

Cilia, noted by Palay (1961) in the preoptic nucleus of the goldfish, and by Taxi (1961) in sympathetic ganglionic cells of the frog and other vertebrates, are prominent in the neurosecretory cells of the toadfish. These cilia are of the 9:0 type which occur, among others, in sensory cells (Barnes, 1961). Two kinds of sensory perception could plausibly be ascribed to cells of the preoptic nucleus: (a) As their homologues in mammals (Verney, 1948), they might be osmoreceptors. (b) They could serve as photoreceptors, in view of the sensitivity to illumination of the diencephalon of blinded fishes (Scharrer, 1928) and the gonadal response of blinded ducks to light directed toward the hypothalamus (Benoit and Assenmacher, 1959).

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July 10, 1962

A common mechanism for temperature adaptation and crossvein deformation in Drosophila. Roger Milkman.

Exposure for 25–40 minutes to 40.5° C. given to *D. melanogaster* pupae 25 hours (at 23° C.) after puparium formation causes posterior crossvein defects in the emerging adults. Longer exposures are lethal. Short exposures, followed by a few minutes at room temperature, increase resistance to defects and to death.

Kinetic evidence indicates that at 40.5° C. a certain protein passes through the sequence of tertiary structures "A," "B," "D," "E," "F," "G." These states have been characterized by their temperature coefficients of formation, temperatures at which significant formation takes place, convertibility at room temperature to a heat-resistant ("C") state, and functionality with respect to crossvein formation.

The lower limit on this type of crossvein defect production, 38.5° C., is set by the "D" to "E" conversion. At lower temperatures a competing reaction, converting "D" to a resistant state (C′), prevents the sequence from continuing to a significant degree.

The individual temperature coefficients ($Q_1 = 1.4$–1.8, depending upon the reaction) imply tertiary structure change but cannot account for the overall Q_1, which is 2.3. This is explained by the competition for "D" also, since the rate of formation of "F" depends both on the concentration of "E" and on the "E" to "F" reaction rate. These results relate a form of rapid temperature adaptation to phenocopying and death via a common path.

The adaptation of Tetrahymena to a high NaCl environment. Philip B. Dunham.

The process of adaptation to a high NaCl environment of *Tetrahymena pyriformis,* a fresh-water ciliate, has been investigated. One per cent of the normal (fresh water) population survived transfer to 200 m*M* NaCl medium. It was shown that this stress tolerance is a heritable character which constituted preadaptive variability in the original population.

Average cell volume decreased with adaptation from 16 $\mu\mu$l. per cell to 10 $\mu\mu$l. However, the amount of dry material per cell remained constant, as shown by the increase in per cent dry weight from 19% for normal animals to 29% for adapted animals.

The main feature of the adaptation was an increased ability to maintain a low cellular NaCl concentration. Cellular Na concentration (Na_i) in normal cells in normal medium (Na_o, 36 m*M*) was 12 m*M*/l. cells. Na_i in normal cells in high NaCl medium (Na_o, 223 m*M*) was 105 m*M*/l. cells. Na_i in adapted animals was 43 m*M*/l. two weeks after starting the culture, and fell gradually to 21 m*M*/l. after 22 months, or 1700 generations. This was selection for ability to regulate Na. Changes in the Na-regulatory mechanism which accounted for decreased Na_i in adapted cells were: (1) the saturation level of Na extrusion increased; (2) apparent free Na space decreased.

The electrophoretic patterns of soluble proteins in polyacrylamide gel from adapted and normal animals were nearly the same qualitatively and quantitatively. However, one protein was in much greater amounts in the adapted animals. Since the two cell types differed only with respect to the response of one to an altered environment, the protein difference reflects adaptive change.

Time-lapse motion pictures of intracellular disturbances induced in Arbacia zygotes after ultraviolet or x-ray irradiation of zygotes, both gametes, or one gamete. CARL CASKEY SPEIDEL AND RALPH HOLT CHENEY.

Strong irradiation, x-ray or 2537 Å ultraviolet, of zygotes, both gametes, or eggs alone, induced violent internal disturbances during the first cleavage cycle in *Arbacia*. Rapid movements of pigment granules and other cytoplasmic constituents occurred, heralding approaching death. The pigment became concentrated in a conspicuous mass, usually centrally located. The nucleus with membrane intact moved about the cell as part of the general upheaval. In contrast, strong irradiation of the sperm alone did not elicit a like reaction. In the resultant zygotes, internal disturbances took place accompanied by sudden gel-sol adjustments, but the pigment granules were distributed in several small aggregations rather than in a large central mass. Massive pigment concentration was correlated, therefore, with irradiation of egg or zygote cytoplasm, but not of sperm.

Ultraviolet effects. Time-lapse motion pictures included: internal upheaval and massive pigment concentration 4–5 hours after fertilization with normal sperm of eggs given 12-minute UV (two examples); similar scenes with eggs *shaken* throughout 12-minute UV irradiation, exposing all sides equally; sperm sticking to exposed half of *unshaken* 12-minute eggs with half fertilization membranes and to entire surface of *shaken* 12-minute eggs with unelevated membranes; highly magnified scenes of UV-induced death throes showing differential viscosity, pigment concentration, nuclear movements, popping gel-sol reactions, and repeated furrow obliteration (two examples); 24-hour delayed development; exaggerated polygonal gelation at border two hours after 4-minute UV to sperm alone; death changes, featured by formation of extra wide hyaline border, 1–2 hours after 12-minute UV to sperm alone.

X-ray effects: adjustments after 3–7 hours, 30–128 kr to zygotes; after 6 hours with furrow obliteration, 120 kr to sperm alone; early death throes with massive pigment concentration within one hour after 60 kr to eggs alone.

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JULY 17, 1962

Organ and ontogenetic patterns of multiple forms of hydrolytic enzymes in Limnaea palustris. JOHN B. MORRILL AND ELAINE N. DOW.

Soluble electrophoretically mobile hydrolytic enzymes of adult organs and 0- to 9-day-old larvae were determined by the method of Hunter and Markert (1957). Adult organ tissues, eggs, embryos or larvae were homogenized, frozen and thawed three times and centrifuged at 10,000 g for three minutes. The supernatants were subjected to starch gel electrophoresis (borate buffer 0.03 M; pH 8.6). Mobile enzymes were developed with the following substrates: α-naphthyl acetate; α-naphthyl acid phosphate; leucyl and alanyl-β-naphthylamide; 6-bromo-2 naphthyl esters of α-glucoside, β-glucopyranoside, β-galactopyranoside, β-glucoronide.

Forty-two electrophoretically mobile bands were developed with extracts of 16 adult organs. Several bands were developed with two substrates. Each organ had its own characteristic enzymatic band patterns with respect to presence, absence and intensity of the bands. No organ had fewer than 10 bands nor more than 32 bands. The liver or digestive gland had the maximum number of bands.

In extracts of 0- to 2-day embryos, 5 enzymatic bands were developed. These bands corresponded to those widely distributed in adult organs. On subsequent days of development additional bands appeared as follows: third day, 4 bands; fourth day, 5 bands; fifth day, 3 bands; sixth day, 2 bands; seventh day, 4 bands; 10-day-old hatched snails, 2 bands. All these bands (total, 29 bands) had mobilities similar to those of adult organs. It is not known when the full complement of adult organ bands is eventually attained. That most of the bands appeared in the embryo during the period of organogenesis and histogenesis suggests these enzymes are associated with the functional differentiation of one or more of the organs in which they occur in the adult snail. The sequential appearance of the several enzyme bands at different stages in development also reflects the direct type of development of this mollusc egg.

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Structural and control genes regulating dopa oxidase activity in Drosophila. Herman W. Lewis.

Genetic analysis of dopa oxidase activity in *Drosophila melanogaster* has revealed that at least four genes interact to produce this activity. One of the genes is involved with qualitative aspects of the enzyme system, the others are involved in the determination of quantitative aspects of the enzyme system. The gene involved in determination of the qualitative nature of the enzyme has been located on the genetic map at 52.4 of chromosome II. A recessive allele at this locus when present in the homozygous condition results in an enzyme system that is thermolabile and has an altered substrate profile relative to the wild type enzyme system. Another gene, located approximately five map units to the right of the above mentioned gene, has a dominant allelic form which when present lowers the dopa oxidase activity 50%. A third gene, also dominant relative to the wild type, has been identified on the right arm of chromosome III. It reduces dopa oxidase activity approximately 35% and its effect is most readily detected when it interacts with a fourth gene located on the right arm of chromosome II. These findings indicate that mechanisms of genetic control of enzyme systems in highly differentiated multicellular organisms may be analogous, if not identical, to the mechanisms demonstrated in microorganisms. The following generalization therefore is probably applicable to all genetic systems. Although at the level of primary events the mode of action of all genes is the same, *i.e.*, the imparting of information for the primary structure of polypeptide chains, from the point of view of the interrelation of their products, genes can be divided into two classes: structural genes, which determine the kind of polypeptide synthesized, and control genes, which are involved in the determination of how much polypeptide is made.

The A and I bands in contracting Limulus muscle. G. W. de Villafranca and C. M. Marschhaus.

The dorsal muscles of *Limulus polyphemus* were tied to splints and glycerinated for at least 10 days. Several bundles were removed at a time, blotted and then blended 45 seconds in a 0.04 M KCl-0.0067 M phosphate buffer (pH 7.4) solution. The fibrils after two washings were photographed under phase contrast with a Bolex 16-mm. movie camera during ATP (10^{-2} or 10^{-3} M) induced contraction. Approximately every hundredth frame (18 frames per second) was enlarged to a final magnification of 1300 or 1700 times. A single, distinctive sarcomere and its striations were accurately measured on the printed series. Muscle stretched prior to glycerination gave better, but not different, results than muscle fixed at rest length. Six complete sequences of different preparations were sharply enough defined to obtain measurements over the total range of contraction exhibited, while in many other fibrils a single sarcomere could be measured at the beginning and end of the contraction.

From initial sarcomere lengths as great as 10.8 μ the fibrils shortened as much as 4 μ (to a minimum length of 5.2 μ, or 3.7 μ when starting from rest length). The A bands shortened as much as 1.8 μ. The major portion of the A band contraction occurred commencing with and continuing below the rest length (sarcomere = 7.5 μ). Decreased I band length accounted for the change in sarcomere size to rest length from the stretched condition. During contraction the A band first becomes more dense at the junction with the I band. It is as though an H zone had opened up but in *Limulus* there is no H zone or M line. Later, during contraction, the central portion of the A band becomes more dense. If only this portion of the A band is considered the total A band, the A band would have shortened to 2.5 μ in a sarcomere of 5.0 μ: that is, the I band would have changed relatively little. Contraction of this muscle does not, in all probability, occur by sliding of I filaments into the A band.

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JULY 24, 1962

On the utilization of C^{14} from glucose for amino acids and protein synthesis by the sea urchin embryo. ALBERTO MONROY AND LETIZIA VITTORELLI.

Unfertilized eggs and developmental stages of *Paracentrotus lividus* were incubated for 60 minutes in 10 ml. of sea water containing 1 μC of C^{14}-glucose (U)/ml. (specific activity 10 μC/mg.). The eggs were then extracted with 10% trichloroacetic acid (TCA). One aliquot of the extract was used for the determination of radioactivity while the largest portion was chromatographed two-dimensionally and the radioactive amino acids identified by radioautography. The insoluble residue was extracted with hot TCA and alcohol-ether. Radioactivity was determined using a liquid scintillation counter. At all stages of development as well as in the unfertilized eggs glucose is taken up and used for amino acid synthesis. The following free C^{14} amino acids have been identified in the TCA-soluble fraction: alanine, serine, glycine, proline, glutamic and aspartic acid. No C^{14} peptides have been found. The rate of uptake in the TCA-soluble fraction rises rapidly following fertilization until the early blastula, then remains constant until the mesenchyme blastula when it starts rising again, and a new peak is attained at the midgastrula stage followed by a decline. On the other hand, incorporation in the proteins only begins after fertilization. The rate of this incorporation increases rapidly until the 32–64-cell stage, then declines somewhat to rise quite steeply again after the mesenchyme blastula stage. A decrease is also observed after the midgastrula. The curve of the incorporation of C^{14}-glucose into the proteins thus duplicates that previously obtained with the administration of radioactive amino acids (Giudice, G., Vittorelli, M. L. and Monroy, A.—*Acta Embryol. Morphol. Exp.* **5**: 113 (1962)).

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Reversible enzymatic reduction of insulin. DEWITT STETTEN, JR., HOWARD M. KATZEN AND FRANK TIETZE.

The hepatic enzyme first purified by Tomizawa has been further studied and found to be a transhydrogenase whereby glutathione is oxidized and the disulfide bonds of insulin are reduced. Coupling of this enzyme with yeast glutathione reductase gave a system wherein TPNH reduced insulin. This permitted a ready evaluation of the K_m for glutathione (8.9×10^{-3} M) and that for insulin (4.3×10^{-5} M). Oxytocin and pitressin could replace insulin but lipoate, cystine and homocystine were ineffective. The enzymatic reduction of insulin is considered to be the initial step in the hepatic destruction of insulin. Its rate may be limited by two negative feedback mechanisms: the known inhibition of glutathione reductase by the phenylalanyl chain of insulin, and the failure of TPNH generation in hypoinsulinism.

Reduction of insulin, whether enzymatic or non-enzymatic, results in the complete loss of detectable physiological and immunological activities of the parent molecule. Reoxidation by oxidized glutathione yields very slight restoration of these activities. However, when this reoxidation is carried out in the presence of hepatic glutathione-insulin transhydrogenase, very considerable recovery of insulin-like activity is observed. Thus, by our test non-enzymatic reoxidation yielded 1.7% recovery of physiological activity whereas in the presence of enzyme 32% was recovered.

The possible role of enzyme-directed thiol-disulfide interchange reactions in the biosynthesis of cystine-containing proteins has been presented.

Golgi apparatus and lysosomes in vertebrate neurons. ALEX B. NOVIKOFF.

In many vertebrate neurons the Golgi apparatus is a large reticular structure as described in 1898 by Golgi. This may readily be observed in frozen sections incubated, by a method developed with S. Goldfischer, for nucleosidediphosphatase or thiaminepyrophosphatase activity. Together with E. Essner, we have studied incubated sections of rat brain (cerebrum, hypothalamus, cerebellum) and cord, and cerebellum of barn owl and pigeon, by light and electron microscopy. In all neurons studied, the reaction product resulting from these phosphatase activities is localized exclusively in the Golgi saccules; none is seen in the ergastoplasm

(Nissl substance). Intimately associated with the Golgi saccules, and apparently derived from them, are numerous granules—"dense bodies" in "typical" neurons and "large granules" in neurosecretory neurons. These granules possess acid phosphatase activity and two other hydrolase activities shown by lysosomes in incubated sections of liver. A small amount of acid phosphatase reaction product is sometimes seen in Golgi saccules. The state of development of ergastoplasm and Golgi apparatus approaches that of neurons only in secretory cells "packaging" materials within Golgi-derived vacuoles; those that we have studied possess fewer Golgi-associated lysosomes than do neurons. The electron microscope evidence and the lysosome distribution, as seen by light microscopy, in the cell processes of neurons—normal and following axon section—lead us to the following working hypothesis. "Secretory" material, produced by the ergastoplasm, reaches the Golgi saccules, perhaps by smooth-surfaced vesicles derived from the ergastoplasm. There it is "condensed" into granules or lysosomes. These move down the cell processes, where their decreasing numbers may reflect release of their hydrolases, at the surface or within the cytoplasm.

JULY 31, 1962

On the chemistry of the thymus gland. ALBERT SZENT-GYÖRGYI AND ANDREW HEGYELI.

The thymus gland contains two biologically active substances, one of which promotes malignant growth, the other which retards it. The former seems to be a specific product of the gland, while the latter is present in various tissues, although at a considerably lower concentration. The activity of the two substances, being antagonistic, can be demonstrated (in inbred Swiss albino mice, inoculated with Krebs-2 tumor) only after they have been separated. Separations can be achieved by paper chromatography.

Thymus glands were extracted with methanol and the active substances precipitated with Reinecke salt. The two substances seem to contain nitrogen, which induces a basic group with a low dissociation constant. The two substances have closely related properties which makes separation difficult. They tend to spread over the various fractions, adhering to any substance present.

The promotor substance sterilizes both male and female mice reversibly. It seems to influence the hormonal background, shifting it towards the pre-puberty condition.

Extracts have been purified several thousand-fold and gave strong biological activity without side effects with dry weights of 0.1 mg. The present extracts allow, thus, biological experimentation.

Effects of heavy water, glycerol and sucrose on glycerol-extracted muscle. BENJAMIN KAMINER.

From previous investigations it was tentatively concluded that the inhibitory effect of heavy water on muscular contraction is due to retardation of the membrane-contractile coupling process. To seek further supporting evidence for this hypothesis, the present investigation was done on preparations of glycerol-extracted muscle which contained an intrinsic relaxing system. This particular preparation was chosen since part of the relaxing system, the sarcoplasmic reticulum, is considered, from work by other investigators, to be involved in the membrane-contractile coupling mechanism.

On soaking the glycerol-extracted bundles in normal water for variable periods of time before the addition of ATP, spontaneous relaxation (and associated responsiveness to calcium) was lost. In heavy water, on the other hand, this relaxing ability was maintained for longer periods of time. It is conceivable, therefore, that heavy water retarded the process which inactivated the intrinsic relaxing system. Conversely, it favored the relaxed state. Whether in fact the heavy water affected the sarcoplasmic reticulum remains, however, to be elucidated.

The properties of water were then altered by the addition of either glycerol or sucrose in varying concentrations. It is well known that glycerol will retard the contraction induced by ATP. In this investigation it was demonstrated, however, that subsequent addition of calcium augments the contraction. Furthermore, prior addition of deoxycholate also led to an augmented response to ATP. In maximally contracted preparations in water-solutions, replace-ment with 25% glycerol led to reversible relaxation. Sucrose had similar effects. In addition, 25% glycerol had a preservative effect on the relaxing property of muscle microsomes (sarcoplasmic reticulum).

Consideration is being given to what extent the altered properties of normal water are involved in all the above effects.

August 7, 1962

Regeneration studies on a brackish-water ciliate, Tracheloraphis sp. Reuben Torch.

Tracheloraphis sp. is a large (500–900 μ) partially flattened, extremely contractile, worm-like ciliate living in brackish water (salinity 10‰). The nuclear apparatus consists of four Feulgen-negative macronuclei clustered around two Feulgen-positive micronuclei, the entire complex surrounded by a mass of small Feulgen-negative granules. Four regions of the animal can be distinguished: head, neck, mid-region, and tail. The black head, slightly wider than the neck, contains the mouth at its anterior tip and is tightly packed with short (2 μ) refractile, birefringent rods. The narrow, extendible neck widens to form the extensive mid-region, which terminates posteriorly in a short, pointed, slightly curved tail.

Dissections were made and the regenerates maintained in depression slides containing small amounts of filtered pond water. The animals were decapitated with glass needles and then cut into halves or thirds. Regenerates were examined for the presence of nuclei, by phase microscopy or after fixation in Champy's or Bouin's, followed by staining with dilute (1:3) Delafield's hematoxylin.

Within 5 minutes after dissection, refractile, 2 μ rods (head granules) accumulate in the anterior parts of all fragments. This is followed by extensions of the anterior regions to form necks. Most fragments (exceptions being anucleate pieces smaller than 150 μ) form necks, but only nucleate fragments form new tails. Mouth parts are difficult to see, but apparent mouth regeneration was observed in several anucleate fragments. Complete regeneration by nucleate fragments occurs within 3–5 hours and is accompanied by a marked increase in body length (2–3 ×). Many anucleate posterior fragments also double in size, but increase in length was never observed in anucleate anterior fragments.

Some evidence suggests that the small, Feulgen-negative spheres on the periphery of the nucleus may have some influence on regeneration. The role of the nucleic acids in regeneration is being investigated by means of radioautography.

"Messenger" RNA and the cell cycle in a fission yeast. Paul R. Gross and John M. Mitchison.

When cells of the fission yeast, *Schizosaccharomyces pombe,* are transferred during exponential growth from a rich malt extract broth medium to a defined minimal medium containing 4.75% ethanol, there is observed a period of about 30 minutes of no growth, *i.e.,* the optical density of the "stepped-down" culture does not change. Following this, growth resumes at the rate characteristic of the new medium (about ⅓ that in the broth). Following stepdown, no net synthesis of RNA can be detected for about one hour, after which it resumes at the new characteristic rate. By labelling with tritiated, C^{14}-labelled, and P^{32} tracers, it is possible to show that both protein and RNA turn over during their respective periods of stasis, with respect to net synthesis. Labelling of protein may proceed at almost 30% of the rate found in controls (*i.e.,* growing normally in the minimal medium). The maximum labelling rate for RNA is about 7% of that in controls. RNA labelled with P^{32} in controls is stable, since no reduction in the radioactivity of a culture sample can be effected by a "chase" of non-radioactive phosphate. That labelled during a stepdown, *i.e.,* the fraction turning over in the absence of net synthesis, is unstable, because its radioactivity diminishes rapidly after the addition of a cold "chase." Base ratio analysis of this RNA shows that its composition is very different from that of ribosomal RNA and transfer RNA, but approaches that expected for a DNA. Thus, the RNA being turned over during a stepdown is probably the "messenger" fraction of this cell, and this may reflect the necessity for reference to the genes concerned with the production of enzyme systems now needed in the new medium. The growth habit and morphology of this yeast permit autoradiographic assessment of the rate of synthesis of RNA at different times in the cell cycle, this without forcing the culture into synchrony (*e.g.*, Mitchison and Wilbur, 1962). This rate rises steadily through the cell cycle during exponential growth, and when the RNA being made is presumably mostly ribosomal. During the stepdown, the rate remains constant through most of the cycle, and doubles abruptly at the end, just prior to the appearance of a cell plate and subsequent fission.

Actin localization in sperm. LEONARD NELSON.

Engelhardt and Burnasheva obtained "spermosin," a substance analogous to myosin, from homogenized bull sperm on prolonged extractions in solutions of high ionic strength. Spermosin splits ATP and combines with muscle actin. Since the contractility of muscular systems depends on the interaction of the complex actin-myosin, a number of investigators have been working on the identification of actin or an analogous protein in sperm. Should the actin-like material be associated with a specific morphological entity of the spermatozoon, its presence could be surmised from its nucleotide-binding capacity. Perchloric acid extracts of washed, sonicated and fractionated bull epididymal sperm flagella show UV absorption maxima at about 260 millimicrons (equivalent to approximately one mole of ATP per 60,000 g. of protein). Immunocytochemical localization of "spactin" was therefore attempted. Rat skeletal muscle was extracted according to conventional procedures for myosin and actin. The purified proteins were injected intravenously into two groups of rabbits. Antisera tested against the antigens yielded single bands in agar gel diffusion tubes. Frozen-dried rat epididymal sperm blocks were incubated for 30 to 60 minutes in the antisera diluted 1:4 in buffer. Sections were examined in the electron microscope. The actin antibodies react with the outer ring of nine longitudinal fibers in the flagellum. The "spactin" seems to be localized in the cortical region of each of the nine outer fibers, while the spermosin, which reacts antigenically at the EM level with myosin antibodies, appears to be confined to the core of the same fibers. It thus appears that in mammalian sperm flagella, there is a differential distribution of substances responsible for generation of the undulatory wave within each of the nine outer fibers, in contrast to the situation in vertebrate striated muscle in which the proteins actin and myosin reportedly occupy separate filaments.

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AUGUST 13, 1962

A possible mechanism for excitation-contraction coupling in crayfish muscle fibers. LUCIEN GIRARDIER, JOHN P. REUBEN AND HARRY GRUNDFEST.

Alternating light and dark bands with a 9 μ periodicity are clearly visible under the light microscope in living unstained isolated muscle fibers of crayfish. During certain procedures the fibers darken and the banding is obscured. Electron micrographs then reveal the formation of vesicles, sometimes $> 4\ \mu$ in diameter and surrounded by a membrane. Two modes of vesicle formation were observed, each produced by specific conditions, but only one, which develops in proximity to the Z lines, is analyzed here.

These vesicles are formed by the swelling of convoluted tubular organelles which originate just under the fiber surface and run radially inward along both sides of the Z lines. They appear to be comparable to the T-system tubules of other muscles. The diameters of these tubules, about 200 Å, permit free movement of ions, but frictional (Poiseuille) resistance to flow of water must be high. The membrane of the tubules appears to be permselective for Cl and positively charged. Since the tubules swell whenever Cl is forced out of the cell the anion must enter the tubules carrying in water by electroosmosis and the water then must be trapped. Particularly clear-cut effects were obtained with intracellularly applied currents. Vesicles developed only with inward currents and only when the intracellular cathode was a KCl-filled microelectrode. Currents in either direction, but applied through a microelectrode filled with K propionate, did not produce vesiculation. In muscle fibers loaded with Cl, long-lasting local currents result when K or Cl in the medium is decreased. Very large vesicles are then formed.

Depolarization of the cell membrane in any manner also produces a local circuit, with the tubules forming one branch of the current path and Cl entering the fiber from the tubules. Thus, during the action potential there will be an outward flow of current through the tubules, causing depolarization of their membranes and an inward flux of anions. A. F. Huxley has suggested that depolarization of the triadic membranes might be the stimulant for releasing an agent that initiates contraction, but no satisfactory means has hitherto been envisaged to cause the depolarization during the action potential. The properties described here allow the tubules to mediate excitation-contraction coupling, since an anionic membrane is effectively in series with the cell membrane.

Water transport and membrane structure in crayfish muscle fibers. John P. Reuben, Lucien Girardier and Harry Grundfest.

Volume changes under a variety of experimental conditions were determined in isolated fibers. In correlation with the membrane potentials the data provided information on water movements initiated by ionic changes under isotonic, hypotonic, or hypertonic conditions, or movements caused by applied currents. Classical osmosis, anomalous or electroosmosis, and water movement associated with metabolic processes could be distinguished. Changes in the structure of the fibers were also observed.

Large volume changes could be produced when the membrane potential was altered while the activity of water was constant. Swelling was always associated with hyperpolarization, shrinkage with depolarization. The effects could be produced by intracellularly applied currents or by altering the ionic environment. Marked swelling occurred when fibers, depolarized and already swollen in a high-K isotonic medium, were returned to the standard solution. The transient depolarization that occurs on removing Cl from the medium was accompanied by a transient shrinkage. The transient hyperpolarization when the Cl was reintroduced was accompanied by swelling. Anomalous water movements were also produced under other conditions that cause large changes in the membrane potential. When ionic changes were made gradually, so as to diminish the electrical driving force, the volume changes due to anomalous osmosis were lessened or abolished.

Anomalous osmosis can arise only from flow of current across charged membranes. Thus, the cell membrane must be so structured as to permit circulation of currents under a large variety of experimental conditions, and must be heterogeneous in structure. The directions of net movements of water indicate that the membrane has a net negative charge with channels of different selectivities. The presence also of sites with positive fixed charges and permselective for Cl is indicated by electrophysiological and pharmacological evidence. Some, and perhaps all, of the latter sites appear to be located in tubular organelles associated with the Z lines and which are probably comparable with the T-system tubules of vertebrate muscle fibers.

August 21, 1962

Studies on the mechanism by which alloxan alters the permeability of islet cell membranes to mannitol. Dudley Watkins, S. J. Cooperstein and Arnold Lazarow.

It has been shown that treatment of toadfish islet slices *in vitro* with 2.5×10^{-4} *M* alloxan (equivalent to a dose of 40 mg./kg.) increased the permeability to C^{14}-mannitol. Since alloxan reacts with sulfhydryl groups, other sulfhydryl reagents have been studied. Tissue slices were pre-incubated in the test solution, washed twice in isotonic saline, and incubated at 0° C. in C^{14}-mannitol. After incubation, the slices were rinsed, weighed and counted.

P-hydroxymercuribenzoate (a monothiol-binding agent) did not affect permeability, whereas both arsenite and cadmium (which at low concentrations selectively bind dithiols) increased the permeability of islet cells. The C^{14} content of control islets was 36% of that in the medium, whereas the C^{14} content of islet slices pre-incubated with 1×10^{-5} *M* arsenite was 62%; higher arsenite concentrations were less effective. When islets were pretreated with 1×10^{-3} *M* to 1×10^{-14} *M* cadmium, the C^{14} content of the tissue was about 50%; when the concentration was increased (to 1×10^{-1} *M*) or decreased (to 1×10^{-18} *M*) no effect on permeability was observed. In contrast to alloxan, which only affected islet tissue (toadfish), arsenite increased the permeability of rat kidney and heart slices as well as toadfish islet. Treatment of tissue slices with 2,3-dimercaptopropanol after pretreatment with either alloxan, arsenite, or cadmium restored the permeability to the control level. Glutathione did not reverse the effect of these agents. These observations are consistent with the hypothesis that alloxan may exert its diabetogenic effect by binding dithiol groups in the beta cell membrane. The arsenite and cadmium effects further suggest that dithiol groups may be of general importance in maintaining the integrity of many other cell membranes as well.

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AUGUST 22, 1962

Evidence against participation of a jelly-splitting agent in sperm penetration of Arbacia eggs. C. R. AUSTIN AND J. PIATIGORSKY.

Dilute suspensions of "dry" *Arbacia punctulata* sperm (1 drop in 3 ml. sea water) were treated with three concentrations (0.02, 0.05 and 0.007%) of the enzyme inhibitor 53 D/k (Parkes, Rogers and Spensley, 1954). The spermatozoa were washed by centrifugation and decantation and added to suspensions of eggs. After 10 minutes the eggs were examined; the proportions found with elevated fertilization membranes were 88% (with 0.007% inhibitor), 64% (with 0.05%) and 11% (with 0.02%). Eggs without fertilization membranes, like those with these structures, had large numbers of spermatozoa at all levels of the jelly coat. Examination by electron microscopy of sections of eggs (fixed with 1.5% $KMnO_4$ solution) showed that the jelly coat had been essentially preserved, and that spermatozoa could be found adjacent to the vitelline membrane in eggs without fertilization membranes. It is inferred that enzyme inhibition had prevented sperm passage through the vitelline membrane but not passage through the jelly coat.

Several attempts to obtain a jelly-dispersing lysin from spermatozoa (as reported by Hathaway, 1960) were unsuccessful when made with purified fertilizin. Sperm suspensions were centrifuged after being treated with purified fertilizin (Tyler, 1949) and the supernatants were added to *Arbacia* eggs. These eggs had been inseminated so that the existence of the jelly coat was shown by the presence of trapped sperms. No jelly loss was observed within one hour (until first cleavage).

In one experiment, spermatozoa were treated with fertilizin containing 100 μg./cc. of fucose, the optimum concentration for discharging the acrosome (Piatigorsky and Austin, 1962). Electron microscopic examination revealed 52% reacted acrosomes (controls 4%). Supernatants from neither fertilizin-treated nor sea-water-treated sperms dispersed the jelly coat.

Aided by Training Grant 2G-998 from the National Institutes of Health.

Neutralization of the fertilization inhibitors in anti-Arbacia-sperm serum by sperm extracts. E. R. BISCHOFF AND C. B. METZ.

Anti-*Arbacia*-sperm rabbit serum can be converted to the non-sperm-agglutinating form by treatment with the proteolytic enzyme papain. Pretreatment of sperm with such univalent antiserum markedly reduces the fertilizing capacity of the sperm (Metz and Schuel, 1961). Sea urchin sperm extracts prepared by freeze-thawing contain at least four antigens. The present study was undertaken to determine whether such extracts neutralize the fertilization inhibiting activity of the antiserum.

Frozen-thawed extracts of 25% *Arbacia* sperm were centrifuged at 10,000 *g* for 10 minutes and added in excess to papain-digested (Porter, 1958) anti-sperm serum and control serum. Increasing dilutions of 1% sperm were treated with these sera in the proportions of one part sperm to two parts serum. The fertilizing capacity of the sperm was measured by the number of eggs cleaved after 1–2 hours. In a typical experiment 4% of the eggs were fertilized by sperm pretreated with the unabsorbed serum, while 100% of the eggs were fertilized when the sperm were pretreated with the extract-absorbed immune serum or control serum. Thus, sperm extracts appear to contain a substance capable of neutralizing the fertilization inhibitors present in the anti-sperm serum.

Since high speed centrifugation of sperm extracts removes most of the egg agglutinating activity (Metz and Köhler, 1960), it seemed of interest to see if the neutralizing substance could also be removed by centrifugation. Univalent anti-sperm serum was absorbed with the supernatant of sperm extracts centrifuged at 30,000 *g* for 30 minutes. The sediment was resuspended in sea water and also tested. Fertilization tests showed that the neutralizing substance was present in the sediment but not in the supernatant.

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Immunological identification of an egg agglutinin in Arbacia sperm extracts. E. R. BISCHOFF AND C. B. METZ.

Sperm extracts that agglutinate eggs can be prepared from sea urchin sperm by various procedures. Metz and Köhler (1960) found that egg-jelly precipitating frozen-thawed extracts of *Arbacia* sperm contain a minimum of four distinct antigens. This suggests that further analysis of the extracts might reveal an identity between one of the four soluble antigens and the egg agglutinin.

When sperm extracts prepared by freeze-thawing are centrifuged at 10,000 *g* for 10 minutes and absorbed with an excess of *Arbacia* fertilizin solution, a pink, jelly-like precipitate forms. Immuno-diffusion of the supernatant in agar against anti-sperm rabbit serum results in the appearance of only three precipitin bands. Evidently one antigen has been removed by the fertilizin treatment. As a further test, sperm extracts treated with fertilizin were used to absorb anti-sperm serum. Since the sperm extract now contains only three antigens, a corresponding number of antibodies should be precipitated from the serum. When such absorbed serum was diffused against normal sperm extract, a single faint band appeared. It is concluded that the fertilizin combines with the egg agglutinin in the sperm extract and removes it as a precipitate.

The control and fertilizin-absorbed sperm extracts were subjected to immuno-electrophoresis in agar gel at pH 8.6. In parallel runs the component which moves the farthest toward the anode is always absent or reduced in the fertilizin-absorbed extracts. In concentrated extracts this arc extends in a continuous band from the origin. This suggests a substance of low solubility or a heterogeneous collection of molecules or fragments which have the same antigenic group.

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Action of neuraminidase on Arbacia spermatozoa. RALPH L. BRINSTER AND C. R. AUSTIN.

Arbacia spermatozoa contain a considerable amount of sialic acid, and Hathaway (1961) has shown that treatment with heat, sodium lauryl sulfate, or fertilizin resulted in the release of a bound form of the substance. He also demonstrated that the antigen of the sperm extract-antibody complex contained bound sialic acid. These observations, together with the known importance of sialic acid as a cell surface component and its role in virus-cell conjunction (Gottshalk, 1957), suggest that it may have an important function in sperm-egg union.

When 4×10^9 spermatozoa were treated for one hour with one ml. of a neuraminidase preparation (crude extract, Sigma), the fertilizing capacity was 99% inhibited, the degree of inhibition showing some variation between different sperm suspensions. In eggs that did undergo fertilization, the enzyme appeared to inhibit cleavage.

The supernatant fluid of the sperm suspensions after enzyme treatment was analyzed for free sialic acid by the thiobarbituric acid method (Warren, 1959), but none could be detected. Furthermore, heating the enzyme to 80° C. for 30 minutes had little effect on its ability to inhibit the fertilizing capacity of spermatozoa. The possibility that the enzyme might be covering sialic acid and/or removing larger molecules containing sialic acid was therefore examined. It was possible to show in preliminary experiments that treatment of spermatozoa with the enzyme preparation did in fact result in the release of bound sialic acid. In addition, incubation of the enzyme preparation for 20 minutes with the supernatant fluid of spermatozoa heated to 60° C. for 5 minutes resulted in a reduction in the ability of the enzyme to decrease the fertilizing capacity of spermatozoa. This supernatant contains large quantities of a bound sialic acid moiety (Hathaway, 1961) and it was confirmed that the substance exercised a competitive inhibiting action.

These observations are interpreted to mean that neuraminidase is capable of removing large molecules containing sialic acid from spermatozoa, and that this reaction can prevent sperm penetration into the egg cytoplasm. A possible general toxic action of the neuraminidase extract on spermatozoa was, however, not completely excluded.

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Passage of spermatozoa through the chorion of Ciona eggs. S. D. EZELL, JR. AND C. R. AUSTIN.

The mature egg of *Ciona intestinalis* is enveloped in a tough membrane, the chorion, which the spermatozoon must penetrate before making contact with the egg proper. Since penetration could well depend on the action of a lytic agent, attempts were made to extract a chorion-dissolving lysin from spermatozoa by freezing and thawing, or by treatment with acidified sea water (pH 4.0–5.0), alkaline sea water (pH 10.5), and "egg water." In no case did the resulting fluid have the capacity to dissolve the chorion. It is inferred that, if such a lysin is indeed present in the spermatozoa, it must exist in very small quantity, or be insoluble in water, or be inactive when extracted.

Study of fertilized and cleaving eggs regularly revealed the presence of many extra spermatozoa within the chorion. These spermatozoa were free within the perivitelline space and active, though not so active as those outside the chorion. Evidently permeability of the chorion to sperms does not change after sperm entry; exclusion of extra sperms from the vitellus presumably depends on a change in the egg cortex.

As seen with the light microscope, the spermatozoon is asymmetric, with a mass of protoplasm of indefinite shape associated with the head region. The presence of this laterally projecting mass posed a problem of special interest in connection with sperm passage through the chorion. Investigation with the electron microscope showed that the mass contained a single large mitochondrion. In freshly shed sperms the mitochondrion appeared to be hemicylindrical and ran most of the length of the nucleus. There was no sign of mitochondrial ("midpiece") structures posterior to the nucleus, as displayed by many invertebrate sperms. The fate of the mitochondrion during chorion penetration has yet to be determined.

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Soluble surface and subsurface antigens of the Arbacia sperm. GORDON P. FLAKE AND C. B. METZ.

Metz and Köhler (1960a, b) found four antigens in extracts prepared by freeze-thawing (Tyler, 1939) *Arbacia* sperm. The present investigation was designed to determine whether any of these are subsurface antigens. The technique employed was complete absorption of sperm agglutinins of rabbit anti-*Arbacia* sperm serum with a 25% suspension of living *Arbacia* sperm, followed by diffusion of the absorbed antiserum against frozen-thawed sperm extract in an Ouchterlony plate. In such experiments two precipitin bands appeared between the extract and the absorbed antiserum, and four bands between the extract and unabsorbed antiserum. Two of the latter joined the two bands of absorbed antiserum. Similar experiments involving urea extracts of *Arbacia* sperm produced one band against absorbed antiserum and two against unabsorbed antiserum. These results indicate that the extraction procedures remove antigens from the *Arbacia* sperm which are not involved in the agglutination reaction and thus are probably soluble subsurface substances.

Treatment of sperm with unabsorbed antiserum rendered univalent by papain digestion (Porter, 1958) prevented their agglutination by egg water, while treatment of sperm with digested absorbed antiserum did not. In addition, pretreatment of sperm with absorbed, undigested antiserum did not prevent their agglutination by egg water. This confirms the fact that the sperm surface antibodies had been removed from the antiserum by absorption with sperm.

Finally, it seemed of interest to examine for an effect of digested absorbed and unabsorbed antisera, as well as undigested absorbed antiserum, on the fertilizing capacity of sperm. Several experiments showed that neither the digested nor the undigested form of absorbed antiserum affected the sperm's fertilizing capacity, while treatment of sperm with the digested unabsorbed antiserum markedly reduced this capacity.

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Electron microscope study of sperm entry into sea urchin oocytes. LUTHER E. FRANKLIN AND C. B. METZ.

Germinal vesicle stages of sea urchin eggs are especially suitable for sperm penetration studies at the electron microscope level because they are normally polyspermic and do not elevate fertilization membranes. Electron micrographs of inseminated *Arbacia* and *Lytechinus* oocytes revealed spermatozoa either just in contact with the egg surface or completely within the cytoplasm. Spermatozoa in the former situation exhibited reacted acrosomes, whereas those in the latter lacked plasma membranes and, usually, nuclear membranes. Exaggerated fertilization cones, characteristic of inseminated sea urchin oocytes, were totally devoid of cytoplasmic organelles, as has been reported in classical literature (Wilson, Harvey). Observations to date have generally agreed with previous studies describing acrosome reactions (Dan) and sperm entry (Colwin and Colwin).

An exceptional case of sperm entry was found in a *Lytechinus* oocyte which had been mildly centrifuged prior to insemination. Serial sections showed a spermatozoon with an intact acrosomal granule pentrating the egg surface at an acute angle. The gamete plasma membranes were closely applied in several regions, but fusion did not seem to have occurred. Engulfment had progressed to the proximal region of the tail.

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Relationship of fertilizin to the acrosome reaction in Arbacia. JORAM PIATIGORSKY AND C. R. AUSTIN.

Collier (1959) and Haino and Dan (1961) observed that fertilizin discharged sea urchin acrosomes and that the frequency of reactions was related to agglutinating titer. This investigation supports these findings and extends the data to *Arbacia punctulata.* Fucose determinations related frequency of acrosome reactions to actual concentration of fertilizin. Fibers of precipitated fertilizin (Tyler, 1949) were washed, redissolved in filtered sea water and dialyzed overnight. Dilute sperm suspensions were treated with serial dilutions of fertilizin of known fucose concentration. The sperms were examined under the electron microscope (JEM 6A).

Acrosome reactions occurred from 10 to 20% with 2–5 μg./cc. of fucose. The reaction frequency increased sharply up to 15 μg./cc. of fucose. The percentage of reactions continued to rise, though less steeply, up to a concentration of 100–125 μg./cc. of fucose, above which there was no significant increase in reaction frequency. Even at this optimum concentration of fertilizin, 100% reactions were never observed, the maxima roughly lying between 50 and 60%. There was large variation between different sperm suspensions, but the relative changes were consistent.

Fertilizin solution boiled at pH 4 for 30 minutes ceased to agglutinate sperms, reducing reactions to percentages only slightly higher than the controls. Preliminary experiments showed that univalent fertilizin, made by gamma irradiation (5000 r/minute) for 5 minutes of a solution containing 20 μg./cc. of fucose, decreased agglutination significantly and lowered the reaction frequency from 50 to 20%. Irradiation for 6–9 minutes left the fertilizin non-agglutinating and primarily univalent. Acrosome reactions were reduced to 3–6%.

These results provide evidence that intact agglutinating fertilizin provokes the acrosome reaction.

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Inducement of the "acrosome reaction" by acridine orange. CHARLES A. SHIVERS AND THOMAS E. EVANS.

Various agents have been shown to be effective in altering the acrosomal region of sperm (Metz, 1957). In a series of observations on spermatozoa of *Echinarachnius parma* and

Arbacia punctulata, employing the dark-field microscope, it was noted that acridine orange (A.O.) caused a morphological change in the acrosomal area that appeared identical with the change induced by exposure of sperm to homologous egg water (E.W.). Ultraviolet (UV) excitation appeared to enhance the reactivity of the A.O.-treated sperm.

Whereas previous reports have dealt with observations of sperm before and after the acrosomal change, this report deals with observations of the reaction as it occurred in the living sperm. This change involved a rapid elongation of the sperm nucleus, accompanied by an apparent disappearance of the acrosomal granule and a slight displacement of the midpiece. These changes in the sperm cannot be mistaken for the cytolysis of sperm heads which results from prolonged exposure of sperm to UV light.

Comparison of electron micrographs of whole mounts of untreated, A.O.-treated, and E.W.-treated sperm of *Echinarachnius* indicated that the A.O.-induced reaction was comparable to the E.W.-induced reaction. Although no quantitative data were taken, it was apparent that the per cent reaction of both the A.O.- and the E.W.-treated sperm was considerably higher than in the controls. In nearly all cases, sperm that appeared reacted in the electron microscope by the criteria presented above (especially the elongation of the nucleus) possessed definite acrosomal filaments.

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Localization of sperm antigens by dissociation of antigen-antibody precipitates. C. ALEX SHIVERS AND C. B. METZ.

An attempt was made to localize sperm antigens with antibody obtained by reversal of antigen-antibody precipitates produced in agar-diffusion plates ("echo technique"; Nace *et al.*, 1960). Localization was followed by indirect staining with fluorescein-conjugated sheep anti-rabbit globulin antiserum.

Anti-*Arbacia* sperm rabbit serum globulins were used. These produce four precipitin bands with frozen-thawed sperm extracts in agar-diffusion plates (Metz and Köhler, 1960). Three of these are strong bands. The weak band was discarded; the strongest of the three remaining (1) and the other two (2) together were cut from the plate and analyzed in the reversal procedure. Controls included agar with antibody alone and with control serum alone.

Isolated bands were adjusted to pH 10–11. After dissociation the pH was readjusted to 7–8 and the dissociated complex layered over air-dried sperm smears. These were treated like tissue sections in the fluorescent technique.

In smears treated with agar and antibody alone, the entire sperm fluoresced intensely. Smears from the dissociated single band (1) showed an intense fluorescence of sperm heads and no tail fluorescence. Fresh sperm treated with this dissociated antibody failed to agglutinate. These observations suggest that the antigen of band 1 is a sub-surface (non-agglutinating) sperm head antigen.

Smears treated with dissociated bands (2) showed fluorescence over the entire sperm. This, plus the fact that fresh sperm are agglutinated by the dissociated antigen-antibody complex of these two bands, suggests that one or both antigens is located on the sperm head or tail or both, and that at least one of the antigens is a surface antigen.

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Axial body and filament formation in oyster sperms. D. H. SPOON, A. FORER AND C. R. AUSTIN.

The unreacted sperm head of *Crassostrea virginica,* seen in dark-field illumination, has a central light region one-third its diameter and less refractile than the head outline. This evidently corresponds to the axial body described from phase-contrast observations and electron micrographs by Galtsoff and Philpott (1960). The axial body lies in a deep anterior depression in the nucleus, and appears unstained by the Feulgen reagent and by the vital nuclear dye, methylene blue. The acrosome granule in dark field is a highly refractile cap on the front of the head.

The acrosome reaction could be observed in individual sperms by introducing under the coverslip oyster egg water, hen egg albumin, or solutions of high alkalinity (pH 9.4) or containing divalent ions (Ca^{++}, Mg^{++}, Mn^{++}). The sequence of events began with swelling of the acrosome granule and loss of its refractility. This was immediately followed by extrusion of the filament, apparently from the axial body, which now became displaced to the widened front surface of the sperm head. With slides and coverslips coated with silicone, filaments did not attach to surfaces and were longer. Depending on the reactant, filaments varied from a stubby extrusion of less than 0.3 μ to a slender rod over 12 μ long.

A high resolution polarizing microscope with rectified lens (A.O. Co.) showed in unreacted sperms at the position of the axial body a region of weak birefringence, with alternate light and dark quadrants at 45° to the angle of the polarizer and analyzer of the microscope. When distilled water was added, the sperm heads swelled and this region moved to the front surface. After filament formation, the birefringence of the region became more diffusely distributed across the front portion of the head. This birefringence seemed to decrease as filament length increased. The filament itself was either light or dark depending on compensator angle. Because the observations were made on structures of a size near the limit of resolution of the light microscope, the interpretation of the birefringence is difficult. However, the birefringence observed in the axial body region of unreacted sperms is compatible with the electron microscopic description of a rod-like object within the axial body parallel to the long axis of the head. The altered birefringence in reacted sperms is possibly attributable to derivation of the acrosome filament from the axial body by a change involving reorientation of a preformed molecular structure.

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Acetylcholinesterase in Mytilus spermatozoa. ARTHUR APPLEGATE AND LEONARD NELSON.

Sperm suspensions from the minced gonads of *Mytilus* are washed with filtered sea water, suspended in pH 7.2 phosphate buffer, homogenized, and then tested for cholinesterase activity by the photometric method of Hestrin (1949). Up to 30% of the substrate is hydrolyzed in 10 hours of incubation at 37° C. The crude homogenate shows optimal esterase activity at 6.0×10^{-3} to 12.0×10^{-3} M substrate concentration, and inhibition at higher concentrations of acetylcholine. V_{max} of the enzyme at substrate concentrations, 0.1×10^{-3} to 10.0×10^{-3} M, is 2.86×10^{-4} μM acetylcholine split/minute/mg. protein, and the K_m is 5.8 $\times 10^{-3}$ M. The enzyme is competitively inhibited by 1.1×10^{-5} M physostigmine.

A partially purified enzyme has been obtained from the sperm homogenates by precipitation with 28% ammonium sulfate at 22° C. This preparation shows optimal activity at 1.0×10^{-3} to 5.0×10^{-3} M acetylcholine and a V_{max} of 4.56×10^{-3} μM acetylcholine split/minute/mg. protein with a K_m of 2.58×10^{-3} M. Preliminary studies with various substrate concentrations indicate that the enzyme splits butyrylcholine at about one-fifth the rate of acetylcholine, and that benzoylcholine is split extremely slowly at concentrations above 6.0×10^{-3} M.

There is no apparent change in flagellar activity of *Mytilus* sperm in the presence of 1.25 $\times 10^{-3}$, 10^{-4}, 10^{-5}, or 10^{-6} M acetylcholine. However as little as 5.0×10^{-5} M physostigmine causes an increase in the rate of normal flagellation.

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Uptake of H^3-thymidine by eggs of Arbacia punctulata. HILDEGARD ESPER.

Experiments were undertaken with H^3-thymidine in *Arbacia punctulata* to obtain information concerning the presence of a precursor of DNA in the cytoplasm of sea urchin eggs. Female sea urchins received two injections each of 20 μC. of H^3-thymidine (specific activity 20 $\mu C./\mu M$), 12 hours apart; eggs were collected after 36 hours. In preliminary experiments eggs were centrifuged in a 0.85 M sucrose density gradient, resulting in separation of the egg into two halves. Data from the liquid scintillation counter showed that the non-nucleated half contained 41% of the activity of the nucleated half, indicating that H^3-thymidine and/or some metabolic product is present in the cytoplasm. Whole-egg homogenates were then extracted by the technique of Ogur and Rosen. Total nitrogen determinations were carried out on aliquots of the whole egg homogenate by the Nessler procedure. Activity has been calculated as corrected net counts per minute per milligram total egg nitrogen. Seventy to 80% of the counts was recovered in the nucleotide fraction, extracted by cold 0.2 N perchloric acid. Only 7% of the counts was found in the DNA fraction, extracted by perchloric acid at 70° C. for 20 minutes. The remaining counts found in the precipitate probably represent unextracted DNA. These data indicate that considerable H^3-thymidine is taken up into the nucleotide pool of the mature egg. The activity of the nucleotide fraction may suggest a synthesis of oligonucleotides. Whether this fraction corresponds to the cytoplasmic DNA precursor previously reported remains to be determined. It is impossible to decide whether the activity of the DNA fraction is due to synthesis by oocytes contaminating the sample or by mature eggs. Preliminary autoradiographic studies have failed to indicate any DNA synthesis by mature eggs.

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Incorporation of C^{14}-glucose into oocytes and ovarian eggs of Arbacia punctulata. HILDEGARD ESPER.

Experiments indicate that C^{14}-glucose is rapidly metabolized by the developing sea urchin oocyte. Two female sea urchins were injected with 0.5 ml. of isotonic KCl to induce shedding of all mature eggs. On the following day these animals were injected with 10 μC. of uniformly labeled C^{14}-glucose (specific activity 20 μC./mg.). After 24 hours the ovaries were removed, fixed in Carnoy's fluid and paraffin-embedded. Six-micron sections were prepared for radioautography with Kodak Nuclear Track Emulsion NTB2, and stored for two to three days before developing. The distribution of tracks indicated different stages of development, probably correlated with yolk synthesis. Small oocytes which had not yet entered a synthesis period were unlabeled. Larger oocytes were densely labeled over both nucleus and cytoplasm, as were a certain number of mature eggs. These latter may have been still in the process of maturation at the time of labeled glucose injection, and subsequently completed their active synthesis period. Mature eggs with no tracks had evidently finished their synthesis period prior to injection of C^{14}-glucose. Extraction by RNase, DNase and hot trichloroacetic acid did not significantly alter this picture, suggesting that the C^{14} from glucose is incorporated primarily into proteins. Numerous grains were found immediately surrounding the eggs, probably indicating incorporation into the jelly coat material.

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Free amino acids and peptides in unfertilized and fertilized eggs of Arbacia punctulata. THOMAS EVANS, ALBERTO MONROY AND ALFRED SENFT.

Chromatographic analyses of the picric acid–soluble components of eggs of *A. punctulata* were carried out, using the Spinco Model 120 amino acid analyzer. In all cases the jelly coats were removed prior to analysis. Large amounts of an asparagine-glutamine fraction, glutamic acid, glycine, arginine, and ammonia were found, as well as lesser amounts of alanine, leucine, isoleucine, valine, methionine, serine, threonine, ornithine, tyrosine, proline, and hydroxyproline, and trace amounts of phenylalanine, histidine, and tryptophan. By comparison of unhydrolyzed and hydrolyzed samples at least five peptides were demonstrated. Calculations of molar ratios of five amino acids were made with reference to the sum molar quantity of those amino acids. Accurate calculations were impossible with the other amino acids, due in some cases (notably the asparagine-glutamine fraction and arginine) to peak asymmetry and in the rest to low concentration (< 0.1 μM). The molar ratios calculated for two runs of unfertilized (UF) eggs and two runs of fertilized (F) eggs (6 minutes and 13 minutes after fertilization) are: glycine, 0.34177 (UF) and 0.44790 (F); alanine, 0.03646 (UF) and 0.06385 (F); glutamic acid, 0.46840 (UF) and 0.40515 (F); isoleucine, 0.10965 (UF) and 0.06046 (F); and threonine, 0.04374 (UF) and 0.02264 (F).

Analysis of a sample of 32-cell stage embryos yielded a pattern similar to the UF and F eggs.

Determinations of total egg N (N_{tot}) and α-amino N (αN) (Moore and Stein, 1954) of 10% trichloroacetic acid extractions were made on both 5-minute F and UF eggs. The ratios obtained of αN to N_{tot} varied somewhat, but indicated a rise in αN shortly after fertilization. Nitrogen determinations on similar amounts of sperm used to fertilize the eggs indicated that very little if any of this change could be attributed to sperm contamination.

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Evidence of a chemotactic substance in the female gonangium of Campanularia. Richard L. Miller and Leonard Nelson.

Fresh sperms released by the male *Campanularia* gonangium were found to swim randomly in a straight line, although the rotation of the head altered the path into a broad spiral. When the sperms reached the vicinity of the opening into the female gonangium, a radical change in the normal mode of swimming was often seen. The frequency of the head rotation increased noticeably in many cases, and there was often a change from movement in a straight line to tight circles and sharp turns. Of great significance, however, was the high proportion of sperms seen to change direction and swim into the female gonangium. Those sperms swimming out of the gonangium were seen to turn back abruptly, but a few left the area completely. This, and the fact that neither agglutination nor a decrease in motility were seen, suggests that more than trap action is involved. In fact, the phenomenon resembles closely chemotaxis as described in ferns and mosses (Rothschild, 1952).

Different portions of the gonangial tissue, as well as sea water and coenosarc tissue controls, were placed in glass capillaries and immersed in a sperm suspension. It appeared that the distal portion of the "funnel" tissue contained the preponderance of the hypothetical attracting agent. The substance seems quite labile in sea water, is permeable to perisarc, and is not sensitive to trypsin digestion unless the cells releasing it are damaged.

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Changes in some proteins in the course of the development of Arbacia punctulata. Ronald J. Pfohl and Alberto Monroy.

Modifications of the protein pattern of the egg in the course of development of the sea urchin, *Arbacia punctulata,* have been investigated by disc electrophoresis. Eggs or embryos were extracted in Tris-glycine buffer (0.2 *M,* pH 8.3), centrifuged at 80,730 *g* and the supernatant concentrated. The separation of the protein components was effected with a current of 2 ma per column for $1\frac{3}{4}$ hours in the cold.

In the unfertilized eggs three bands which stained heavily with amido schwarz and seven to nine fainter bands were observed. Aside from a slight decrease in the intensity of staining and the variable disappearance of some of the fainter components, there was no change in the amido schwarz staining band pattern in the course of development.

In the unfertilized eggs, three bands, the fastest one corresponding to the fastest amido schwarz component, showed esterase activity. In the early pluteus stage, the middle component split into two bands, neither of which corresponded to an amido schwarz band. In the late pluteus, five bands with esterase activity were present. The two new components corresponded to the two slowest amido schwarz bands. In general the esterase activity was considerably more intense in the developmental stages than in the unfertilized eggs.

Acid phosphatase activity was distributed in a minimum of four bands. There was no distinct increase in activity in the developmental stages over the activity in the unfertilized eggs. The alkaline phosphatase activity in the unfertilized eggs was weak and present in one band corresponding to the fastest amido schwarz component. At the mesenchyme blastula and later stages, a substantial increase in activity was apparent, thus confirming the observations of Mazia *et al.* (*Biol. Bull.,* **95**: 250). The activity was almost entirely localized in a new, more slowly moving, component, distinguishable from the former faint band.

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Electrophoretic and ultracentrifugal analysis of the fractionated extracts of Arbacia punctulata eggs and early plutei. Ronald J. Pfohl and Alberto Monroy.

By the use of disc electrophoresis, changes have been described in the protein pattern of the egg in the course of development of *Arbacia punctulata* (Pfohl and Monroy, previous abstract). Extracts prepared as described have now been fractionated by precipitation at 45% (45 fract.) and saturation of $(NH_4)_2SO_4$ (sat. fract.). Precipitates were analyzed, using disc electrophoresis (DE) and analytical ultracentrifugation (UC).

No differences were detected in the UC patterns of the total extracts and fractions thereof between unfertilized eggs and early plutei. The 45 fraction exhibited three components with the following S_{20} values: (1) 1.22–1.36; (2) 1.98–2.70; (3) 7.08–7.21. The sat. fraction had two main components with S_{20} values of (1a) 5.32–5.77; and (2) 8.38–9.29. A small component in the latter formed a shoulder of (1a) and seemed to correspond to component (3) of the 45 fraction.

In the DE patterns the slowest, heavily staining (amido schwarz) components were present in the saturated fraction, whereas the heavily staining, fastest component was of about equal intensity in both fractions. All of the esterase activity and most of the acid and alkaline phosphatase activity corresponding to the latter band was present in the sat. fraction. The middle esterase band of the unfertilized eggs, which is split into two bands in the early pluteus stage, is almost entirely present in the 45 fraction.

No correlation is as yet possible between the UC and DE patterns. It seems, however, worthwhile emphasizing the following points: (1) the esterase and phosphatase activities of the fast DE band are almost entirely precipitated above 45% of $(NH_4)_2SO_4$, whereas the part precipitated at 45% appears to be devoid of such enzymatic activities; (2) the esterase activity is present in three main bands, showing apparently the same substrate specificity; by fractional precipitation, however, they can be separated into two groups.

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An actin-like protein isolated from starfish sperm. KENT M. PLOWMAN AND LEONARD NELSON.

An actin-like protein, "spactin," was prepared from sonicated *Asterias forbesii* sperm from which about 80% of the heads had been removed by centrifugation. The suspension of flagella and midpieces was extracted according to the actin isolation of Tsao and Bailey and of Mommaerts, yielding a clear, gel-like pellet.

Analysis of a 5% perchloric acid extract of the protein by paper chromatography, with n-butanol-ammonia, yielded a single ultraviolet-absorbing spot which matched the ATP controls in R_f values. The eluate had an absorption spectrum identical with ATP. By assuming a molecular weight of 60,000, one can estimate from dialyzed preparations that 0.6–1.4 moles of ATP were bound per mole of protein. The spactin pellet was soluble in distilled water and "salted-out" at ionic strengths above 0.05 KCl. Water solutions appeared somewhat thixotropic in the Ostwald viscometer. When run in the analytical ultracentrifuge, a fresh sample in distilled water produced a single major peak, although an older sample gave two peaks of equal size. When the peak had sedimented nearly to the bottom of the cell, 90% of the nucleotide was associated with it. When one part of spactin was combined with three parts of purified rabbit myosin in a final concentration of 0.4 *M* KCl, a precipitate formed on addition of 10^{-3} *M* ATP. The specific activity of this precipitate, redissolved in 0.5 *M* KCl, as an ATP-ase, was 50% of that of the rabbit myosin in $CaCl_2$ at pH 8.9 and 7.2 and $MgCl_2$ at pH 7.2, but was nearly equal in activity to that in $MgCl_2$ at pH 8.9. This solution had two very sharp peaks in the ultracentrifuge, with Svedberg constants of 4.5 and 6.5 at 0.42% protein concentration.

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GENERAL SCIENTIFIC MEETINGS

AUGUST 27–30, 1962

Abstracts in this section (including those of Lalor Fellowship Reports) are arranged *alphabetically by authors* under the headings "Papers Read," "Papers Read by Title," and "Lalor Fellowship Reports." Author and subject references will also be found in the regular volume index.

PAPERS READ

/ [illegible] papers

Differentiation of synaptic and GABA inhibitory action in crab neuromuscular junctions. EMILIO ALJURE, HAROLD GAINER AND HARRY GRUNDFEST.

Intracellular recording from the adductor muscle of the dactylus of walking legs of *Cancer borealis*, together with selective stimulation of excitatory and inhibitory axons, confirmed that GABA does not activate inhibitory post-synaptic membrane. At 10^{-4} w/v, GABA blocks epsps reversibly without decreasing the effective resistance of the muscle membrane, whereas stimulation of the inhibitory axon at 100/sec. decreases the effective resistance by 30%. GABA does not appear to inactivate the excitatory post-synaptic membrane, since the amplitude and frequency of spontaneous "miniature" epsps are unaltered by its application. No effect of GABA on the non-synaptic membrane of the muscle fibers was observed. It did not alter the rates of movements of K^+ and Cl^- as determined from the responses of the membrane potential to changes in outside concentration of the respective ions. Thus, GABA acts on the presynaptic terminals. Furthermore, combinations of picrotoxin with serotonin or phenylethylamine (10^{-4} w/v each) cause repetitive antidromic discharge of the axons in response to a single orthodromic impulse. As in lobster, the firing appears to be due to sustained depolarization of the axon terminal. This repetitive firing is blocked by GABA (10^{-4} w/v).

Synaptic inhibition of the epsp appears to be greater than can be accounted for by the conductance increase of the post-synaptic membrane. It seems likely, therefore, that the inhibitory transmitter has a dual action: (1) to activate the inhibitory synaptic membrane, and (2) to block the epsp. The latter might be due to interference with the excitatory transmitter or with its release.

Studies on thrombocytes of the smooth dogfish, Mustelis canis. FRANK A. BELAMARICH, RUSSELL F. DOOLITTLE AND DOUGLAS M. SURGENOR.

Thrombocytes of the blood of vertebrates have been implicated in the clotting mechanism, but only the mammalian thrombocyte (platelet) has been extensively studied. The latter is not only involved in clot promotion, but is necessary for clot retraction. ATP and serotonin are released from platelets during the clotting process.

When white cells of the dogfish are added to plasma, clotting activity is increased. No clot retraction takes place unless white cells are present. Two general populations of white cells could be achieved by density gradient centrifugation. Granulocytes are the main component of the layer above 0.75 *M* sucrose, non-granulocytes collect over 1.0 *M* sucrose, and the red cells accumulate at the bottom. When the two populations of white cells were tested for clot-promoting activity they exhibited no differences.

White cells incubated with plasma containing C^{14}-labeled serotonin exhibit a gradual increase in the uptake of label over a three-hour period. If, after this time, plasma containing no labeled serotonin is substituted, the labeling decreases at a rate comparable with the rate of uptake. Since the initial rate of uptake is near the rate of diffusion, it is concluded that thrombocytes do not actively accumulate serotonin, nor do they retain it against a concentration gradient.

Whole blood, plasma, serum, red cells, and white cells were tested for smooth muscle-stimulating activity on a section of dogfish pyloric stomach. Serum had slight activity, while a crude extract of white cells had a high degree of activity. When the crude extract was chromatogrammed (n-butanol/acetic/water, 60:15:25) there were a number of ninhydrin-positive areas as well as one UV-absorbing and one UV-fluorescing area. Only slight activity could be recovered after chromatogramming, and no conclusion can be made at this time concerning the chemical nature of the smooth muscle stimulator.

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On the nature of dogfish trypsinogen and trypsin. DAN C. BRYANT, RONALD S. WEINSTEIN, DAVID L. KLEIN AND R. F. DOOLITTLE.

The purpose of this study was to find what changes have occurred in the trypsinogen molecule in the course of evolution. In all experiments tosyl-L-arginine methylester (TAMe) colorimetric assays for trypsin activity were used. The pancreas of the spiny dogfish (*Squalus acanthias*) was shown to contain an enzyme similar in its specificity to mammalian trypsin. Spiny dogfish pancreases were homogenized in $CaCl_2$ solutions buffered at pH 8.0 with Tris, the homogenates centrifuged, and the resulting supernatant fluids kept at 2° C. During the course of several days, precipitates formed and were removed by centrifugation, leaving a solution which showed twice as much trypsin activity per gram protein as commercially prepared "purified" hog trypsin. Attempts at further purification indicated that both dogfish and hog trypsin are salted out in the 40 to 70% range of saturated $(NH_4)_2SO_4$. Comparison of dogfish with crystalline bovine trypsin indicated that the dogfish trypsin preparation was far from crystalline purity. Heat inactivation experiments carried out in the 46° C. to 60° C. range demonstrated that elasmobranch trypsin is more thermo-labile than hog trypsin. When incubated for 5 minutes at 56° C., dogfish trypsin retained 10% of its original activity, while hog trypsin retained 70% original activity. The autocatalytic conversion of dogfish trypsinogen to trypsin was studied in preparations in which native trypsin was inhibited with diisopropyl phosphofluoridate (DFP). Aliquots of dogfish trypsinogen were inoculated with dogfish trypsin and hog trypsin solutions of varying activities. Dogfish trypsin catalyzed the formation of trypsin from dogfish trypsinogen at a greater rate than did hog trypsin. It is concluded that dogfish and hog trypsin molecules are similar in that both (1) are inactivated by DFP, (2) hydrolytically split TAMe, and (3) have similar salting out properties. In contrast, molecular differences are indicated by differences in temperature stability and the species specificity of the trypsin-initiated conversion of trypsinogen to trypsin.

Purification and some properties of lipoyl dehydrogenase from dogfish liver. C. P. Channing, A. Eberhard, A. H. Guindon, C. Kepler, V. Massey and C. Veegar.

Lipoyl dehydrogenases isolated previously from other sources (pig heart, beef liver, *E. coli,* spinach leaves) are flavoproteins in which catalysis depends not only on the flavin but on another prosthetic group, a protein disulfide linkage. Lipoyl dehydrogenase from the dogfish (*Squalus acanthias* and *Mustelus canis*) has now been isolated and its properties compared with those of the pig heart enzyme. A 1000-fold purification was achieved by extraction with dilute salt, (0.03 *M* phosphate, pH 6.3, $+3 \times 10^{-3}$ *M* EDTA + 2% (w/v) $(NH_4)_2SO_4$), heating to 80° C. for ten minutes, adsorption on and elution from calcium phosphate gel, $(NH_4)_2SO_4$ fractionation between 0.55 and 0.85 saturation, and column fractionation with calcium phosphate gel and diethylaminoethyl cellulose. The resulting enzyme was yellow, showing absorption maxima at 453 mμ and 340–360 mμ and with a pronounced shoulder at 480 mμ. Like the pig enzyme, the dogfish lipoyl dehydrogenase is also extremely fluorescent, a property almost unique among flavoproteins where the fluorescence of the flavin is generally quenched on binding to the protein. The fluorescence excitation spectrum has peaks at 290 mμ, 360 mμ and 460 mμ, and the emission spectrum is maximal at 518 mμ. Evidence has also been obtained indicating the catalytic functioning of a disulfide prosthetic group. Incubation studies in the presence of 10^{-3} *M* arsenite show that inhibition is obtained only when DPNH is included in the incubation mixture; inhibition is not obtained on incubation with DPNH or arsenite alone. Thus it appears that a disulfide is reduced to a dithiol by reducing substrate. A further comparison of the reaction mechanisms of lipoyl dehydrogenases from various sources is under investigation.

Studies on the dissociation of Loligo pealei hemocyanin. L. B. Cohen and K. E. Van Holde.

The hemocyanin of the squid *Loligo pealei* has been studied by sedimentation and diffusion measurements on blood diluted with 0.1 ionic strength buffers. Four main sedimenting boundaries have been observed between pH 6.1 and 10.7: (A) a homogeneous substance of molecular weight 3,400,000, as determined by sedimentation and diffusion, and sedimentation coefficient $S_{20}^{\circ} = 56.1$; (B) and (C), components usually observed together, with $S_{20} = 19$ and 13, respectively, and (D) another homogeneous material of molecular weight 270,000, determined by analysis of boundary spreading in sedimentation, and $S_{20} = 10$. In the absence of added

Mg^{++} ion, (A) is the principal component below pH 7.2, at which point dissociation into (B) and (C) occurs overnight. Between pH 7.6 and 9.1 (B) and (C) are present. At pH 10.0 and above (D) alone is found. A substance apparently identical with (D) was found as the only component in a solution containing 3 *M* urea at pH 6.6. In 0.01 *M* Mg^{++} concentration (A) is stable to pH 9.9, at which point dissociation into (D) occurs. This process is reversible if 0.01 *M* Mg^{++} is present.

The apohemocyanin prepared by removing the copper by dialysis against KCN solution exhibits a sedimentation pattern identical with that of the native protein below pH 7.8 in the presence of 0.01 *M* Mg^{++} ion. At higher pH, dissociation of (A) occurs first with a gradual decrease of S, and eventually entirely into (B) and (C). Above pH 9 component (D) is again found. The dissociation at pH 8.4 has been found to follow first order kinetics by observing the change in boundary area in an ultracentrifuge experiment. Apohemocyanin, which had been taken to pH 8.4 for 56 minutes, exhibited partial reassociation when stored overnight at room temperature at pH 7.2. In this reassociated apohemocyanin we have seen a 36S component.

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Spectral studies of hemocyanin. H. A. DePhillips, Jr. and K. E. Van Holde.

An investigation of the spectra of the hemocyanins from *Busycon canaliculatum* and *Loligo pealei* has been carried out. Blood from *Loligo* was diluted directly with 0.1 ionic strength buffers; the *Busycon* hemocyanin was purified by dialysis and ultracentrifugation. A preliminary study of the pH-stability diagram of the *Busycon* hemocyanin was conducted. Eriksson-Quensel and Svedberg (1936) report that the principal component in diluted *Busycon* blood between pH 4.6 and 7.7 was of sedimentation coefficient 100S; above this pH, partial dissociation into 60S material was observed. At pH 9 complete dissociation into 13S material was found. We have been able to confirm these results *only* if the solutions of purified hemocyanin were made 0.01 *M* in Mg^{++} ion. In the absence of added Mg^{++} the hemocyanin undergoes each dissociation at a lower pH value. Also, we have observed dissociation at pH 3.8; when 0.01 *M* Mg^{++} is present, boundaries of S_{20} equal to 96.2, 81.3, and 10.5S are observed. In the absence of Mg^{++} 82.8S and 10.5S material is found.

Measurements of the absorption spectra in the range 300 mμ to 700 mμ were carried out with oxygenated and deoxygenated *Busycon* hemocyanin, and with the oxygenated hemocyanin and apohemocyanin from *Loligo*. Both oxygenated materials show maxima at 345 mμ and 570–580 mμ. The optical densities of the deoxygenated *Busycon* hemocyanin and the apohemocyanin from *Loligo* exhibit a smooth increase with decreasing wave-length, presumably due to scattering. The curve from the deoxygenated *Busycon* hemocyanin, which was linear in $1/\lambda^4$ between 330 mμ and 700 mμ, was subtracted from the curve for oxygenated material. The dry weight of the *Busycon* material was determined. Extinction coefficients were 593 l/mole-cm. at 570 mμ, and 14,650 l/mole-cm. at 345 mμ, on the basis of copper molarity, assuming 0.25% copper. The optical rototory dispersion of the *Loligo* hemocyanin has been studied between 300 and 700 mμ. Both of the bands exhibit negative Cotton effects.

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Inhibitors of lobster blood clotting. R. F. Doolittle and L. Lorand.

The plasma of lobsters and certain other crustaceans is known to contain a soluble protein ("fibrinogen") which is capable of forming a clot. This conversion is effected by a thermolabile, non-dialyzable factor present in various lobster tissues and blood cells and is calcium-dependent. The finding that lobster clots are insoluble in 5 *M* urea and in 1% monochloroacetic acid suggested that the crosslinks formed during clotting were covalent in character and possibly analogous to those formed in vertebrates by the combined action of thrombin and fibrin-stabilizing factor. The fact that papain can induce similar clots in mammalian systems by acting directly on fibrinogen (*Biochem. Biophys. Res. Comm.*, **7**: 457, 1962) strengthened such a hypothesis. Since crosslinking of mammalian clots can be inhibited by some glycine amides and esters (*Nature*, **194**: 1148, 1962), a similar study of potential inhibitors of lobster (*Homarus americanus*) clotting was undertaken. Lobster blood coagulation induced by the addition of homologous muscle extract was appreciably inhibited by glycine methylester and glycylglycine methylester at concentrations of less than 0.2 m*M*. The specificity of the inhibition is apparent from a quantitative scaling of the degree of retardation based on the concentration of inhibitor necessary for a five-fold increase in clotting time. The best inhibitors found thus far were arbitrarily set to 100: glycine methylester (100); glycylglycine methylester (100); L-lysine ethylester (12); glycineamide (6); DL-serine methylester (1.7); L-tyrosine methylester (0); L-isoleucine methylester (0); L-histidine methylester (0); tosyl-L-arginine methylester (0); glycylglycine (0); glycine (0); methyl acetate (0); lysine (0); epsilon-aminocaproic acid (0). Clotting was also completely inhibited by very low concentrations of p-mercuribenzoate, iodoacetate and cupric chloride, indicating the importance of functional sulfhydryl groups. Hydroxylamine was also found to be a good inhibitor.

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Electrical activity associated with bioluminescence in a single cell. ROGER ECKERT.

Single luminescent *Noctiluca* (Eckert and Findlay, this issue) were held in sea water to the end of a small horizontal pipette by means of slight hydrostatic pressure, and were positioned over the objective of an inverted-type compound microscope. A selector prism in the base of the microscope could be used to divert the image of the cell from the oculars to the photocathode of a multiplier tube. Stimulating pulses were passed between the lumen of the suction pipette and the bath. A recording capillary electrode was inserted through the thin peripheral cytoplasmic layer into the large internal vacuole. Stimulating current, potentials, and light flux were simultaneously recorded and displayed.

An all-or-none 40–70 mv negative-going spike was recorded in response to sufficient current. It reached its peak in about 7 msec. and subsided in another 4 msec. The emission of light begins at about the time of peak potential, and is never recorded in its absence. The light flash reaches its maximum intensity in about 20 msec. and decays to 50% in another 20 msec. Repetitive stimuli with 100-msec. intervals elicit facilitation of the luminescent flashes, while stimulation at intervals shorter than the duration of each flash brings about summation of flashes as well. Neither phenomenon is accompanied by facilitation or summation of the action potentials.

Further evidence that the intensity of the flash is independent of the potential size *per se* was obtained by the addition of KCl to the bath in amounts large enough to diminish the amplitude of the action potential. In that case the flash intensity remained unaltered as successive action potentials became smaller. Only when there was no longer any sign of an active electrical response did the emission of light suddenly fail.

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Nutrient transport in the starfish, Asterias forbesi, as studied with isolated digestive glands. JOHN CARRUTHERS FERGUSON.

Individual digestive glands, weighing approximately 0.6 gram, were removed from healthy starfish and placed in 25 ml. of either filtered sea water or pooled, cell-free coelomic fluid to which small amounts of high specific activity glucose-C^{14} or glycine-C^{14} had been added. The preparations were gently aerated and maintained at 21° C. Samples of fluid were taken periodically for 10 hours and activity assayed. Little difference was observed in the mean rate of removal of glucose-C^{14} from sea water (half-time, 2.16 hours) and coelomic fluid (half-time, 2.26 hours). Glycine-C^{14}, however, was removed rapidly from sea water (half-time, 1.76 hours), but its rate of disappearance was somewhat inhibited in coelomic fluid (half-time, 4.73 hours). Assuming exchange rather than simple removal, the total amount of glycine (or possibly other amino acids) in the coelomic fluid would be turned over in 6.8 hours. Relating this value to conditions in the animal would indicate a turnover time *in vivo* of 0.6 to 0.7 hour. This high rate of movement of amino acids through the coelomic fluid would enable it to function effectively as the medium of transport in spite of its low content of nitrogenous substances, for which a mean value of 63.5 μg. N per ml. has been determined. When two digestive glands were placed in 25 ml. of sea water, nitrogenous substances appeared in the water at a rate that decreased with time, apparently approaching an equilibrium at a concentration of 30 μg. N per ml. These results confirm the tracer studies and indicate that nitrogenous substances are constantly diffusing from the digestive glands at fairly steady rates, but are being reabsorbed at rates dependent on their concentration in the surrounding medium and on the physiological state of the tissue. It seems probable that similar phenomena, differing only in degree, are occurring in all of the tissues of the starfish.

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Effect of temperature on polymerization of G–ADP actin. ROBERT J. GRANT.

Since G–ADP actin polymerizes simply upon the addition of KCl and Mg, without the addition of ATP and its subsequent dephosphorylation to ADP, it provides a simplified material for the study of actin polymerization.

That the polymerization of G–ADP actin to F–ADP by KCl and Mg has a higher temperature coefficient than the polymerization of G-ATP by the same reagents is shown by the fact that at 29° C. these reagents cause both proteins to polymerize in similar fashion, while at 1° they will cause the polymerization of G-ATP but not that of G-ADP. This suggested that the polymerization of G-ADP is an endothermic process capable of being reversed simply by a temperature change. As a prelude to further thermodynamic characterization of the G-ADP polymerization process, an attempt was made to demonstrate this reversibility.

If salt is added to a G-ADP-Mg solution at 1°, no polymerization occurs, *i.e.*, there is no viscosity rise. If the solution is transferred to 29° there is a rapid rise in viscosity, denoting the formation of polymers. After returning the polymerized material to 1° the high viscosity falls off. The rate and extent of this reversible depolymerization are dependent on the pH, the buffer used, and the concentrations of protein, KCl and $MgCl_2$. That the protein is still active, *i.e.*, that true reversal has been effected, is shown by the recovery of high viscosity upon returning the depolymerized G-ADP to 29°. In this way, 80–90% reversible depolymerization has been achieved.

Further studies will be undertaken to determine the equilibrium constants of the polymerization at various temperatures and the entropy changes involved.

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Influence of aldehyde chain length on the relative quantum yield of the bioluminescent reaction of Achromobacter fischeri. J. W. HASTINGS, J. A. SPUDICH AND G. MALNIC.

Light emission using highly purified bacterial luciferase requires $FMNH_2$, oxygen, and long-chain aldehyde, the sequence of reaction being in that order. Added aldehyde is not necessary for the reaction of enzyme with $FMNH_2$ or for the subsequent oxidation. However, the amount of light emitted upon oxidation of the reduced enzyme intermediate is considerably greater in the presence of aldehyde. Since with aldehyde initial light intensity (I_o) is greatly increased, without a proportionate increase in the decay constant (k), the effect of aldehyde may be formally described as an effect upon the quantum yield of the oxidation of reduced enzyme. It was of interest, therefore, to determine both I_o and k for different aldehydes, and to evaluate the relative quantum yield.

Experiments were carried out at 24° C. with pure luciferase, 0.05 *M*, pH 6.8 phosphate buffer, and $FMNH_2$ reduced with H_2 with platinized asbestos.

The aldehydes (either obtained commercially or synthesized by a $LiAlH_4$ reduction) were purified immediately before use by gas chromatography and used as a water-saturated solution, excess droplets being removed by centrifugation. Measurements at different aldehyde concentrations (with 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 18 and 20 carbons) showed typical saturation curves, having characteristic values for both I_o and k with each aldehyde. I_o and k vary regularly with carbon chain length, showing three maxima, at 4, 9 and 14 carbons. The relationship between I_o and k is such that there occurs a parallel but much less accentuated variation in quantum yield. The highest and approximately equal values for quantum yield were obtained with aldehydes having 12, 13 and 14 carbons, lower values being measured with aldehydes having more or fewer carbon atoms. The maximum quantum yields obtained were approximately 45 times those obtained without added aldehyde.

Hemerythrin: dissociation into subunits and reconstitution. IRVING M. KLOTZ AND STEVEN KERESZTES-NAGY.

The molecular weight of hemerythrin, 107,000, has been evaluated by several hydrodynamic and thermodynamic methods. This molecule contains 16 Fe atoms, and hence 8 oxygen-binding sites, since it has been established previously that each O_2 is held by 2 Fe. It seemed of interest, therefore, to see if the native macromolecule was constituted of subunits.

Such subunits, of experimentally determined molecular weight near 14,000, were obtained first by each of three methods: exposure to high pH in Na_2CO_3 solutions; exposure to an anionic detergent, sodium dodecyl sulfate; conversion of cationic lysine side-chains to anionic ones by reaction with succinic anhydride. These treatments are relatively strong, however, and irreversible.

It has also been found recently that hemerythrin contains 1 SH group per 2 Fe atoms. We have now discovered that this SH group plays a vital role in maintaining the size of the native macromolecule. If a mercurial, salyrgan, which combines with SH groups, is added to hemerythrin, the macromolecules are dissociated into subunits of 14,000 weight; at an SH/Fe ratio of 0.5, dissociation is complete.

The subunits will reaggregate spontaneously into methemerythrin if the mercurial is removed from the protein by addition of a mercaptan. With cysteine ethyl ester, added in 5:1 ratio to the Fe, reconstitution was complete. Furthermore, chemical reduction with $NaBH_4$ plus $NaHSO_3$, followed by admission of oxygen, regenerated the red-violet color of oxyhemerythrin.

These investigations were assisted in part by a research grant (H-2910) from the National Heart Institute, United States Public Health Service, and grants from the Graduate School Research Fund of Northwestern University.

Effects of D_2O on the cortical gel structure and cleavage capacity of Arbacia eggs. DOUGLAS MARSLAND, ARTHUR M. ZIMMERMAN AND HARVEY ASTERITA.

Previous work has shown that a substitution of D_2O for H_2O in sea water, to the level of 70% or more, stops all activity in the mitotic apparatus, but does not stop the furrowing process —provided the eggs have approached to within about two minutes of cleavage time when the treatment is applied. The current work represents an attempt (1) to evaluate the effects of lower concentrations of D_2O upon the intensity of the furrowing process, as judged by its resistance to blockage by high hydrostatic pressure, and (2) to relate these observations to pressure-centrifuge measurements of the gelational state of the cytoplasmic cortex.

Eggs immersed in 5% D_2O artificial sea water, starting 40 minutes after insemination, reached the furrowing stage in synchrony with control eggs in non-deuterated artificial sea water. The intensity of the furrowing process was slightly but consistently greater in the deuterated eggs, tested with reference to their capacity to maintain their furrows when exposed to high pressure (5000 lbs./in.^{2} at 20° C.). The pressure, applied at the time when 20% of the eggs displayed incipient furrow and maintained for 20 minutes, caused complete suppression of the furrows in only 47% (±4) of the deuterated eggs, as compared to 64% (±3) of the controls. Higher concentrations of D_2O could not be used, since more heavily deuterated eggs were retarded and asynchronous in their approach to the cleavage stage.

No measurable effect of 5% deuteration upon the gelational state of the cytoplasmic cortex could be observed in eggs subjected to pressure-centrifugations (8000 lbs./in.^{2}; 41,000 g; 20° C.) performed 10 minutes prior to cleavage, after 20 minutes of immersion. In fact, to obtain a measurable stiffening of the cortical gel, as judged by an increased resistance to the displacement of the cortical pigment bodies, it was necessary to raise the D_2O content of the sea water to 40%.

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Contrasts in activation of the egg of Arbacia punctulata. ARTHUR K. PARPART AND THOMAS V. N. BALLANTINE.

In the past, artificial activation of eggs of *Arbacia punctulata* has been brought about by non-electrolytes that penetrate the cortical granules, by osmotic shock, by detergents, by acid and alkali. Each of these, as do sperm, causes cortical granule explosive breakdown.

Present studies show that cysteine in sea water artifically activates eggs without breakdown of cortical granules. Activation was 100% at 0.005 *M* and 80% at 0.001 *M* cysteine. All eggs were re-exposed to sea water at 5 minutes. Activation was normal: the subsurface "cortical gel" gelled in 3 minutes and contained the usual increase in number of motionless echinochrome granules; the cytoplasmic changes for centering the nucleus; the breakdown of the nuclear membrane and "streak stage" all occurred. The cleaved (25%) and uncleaved eggs had a good cortical granule layer around them. Glutathione gave less activation.

In contrast to cysteine, the enzyme papain (2 × crystallized) caused normal explosion of the cortical granules (1 minute), egg activation and cleavage at a concentration of 8×10^{-6} *M* in the presence of any one of the following, in 0.001 *M* concentration: cysteine, glutathione, EDTA, ascorbic acid and KCN. There was a good tight fertilization membrane and hyaline layer around eggs activated by papain! EDTA, ascorbic acid and KCN didn't cause breakdown of cortical granules or activation. Trypsin, chymotrypsin or hyaluronidase with EDTA had no effect. It appears that papain, unlike trypsin, has no effect on the vitelline membrane, but it does affect the membrane of the cortical granules, leading to the release of the glycoprotein present inside the granule. It is believed that the action of cysteine is correlated with its effect on the degree of gelation of the interior of the egg.

Separation of phasic and tonic contractions in Spisula intestine. C. L. PROSSER, D. MARTIN AND R. SHA'AFI.

The longitudinal smooth muscle of intestines of bivalves *Mercenaria* and *Spisula* shows both phasic and tonic contractions. Spontaneous phasic contractions and those resulting from electrical stimulation are accompanied by fast action potentials; no electrical accompaniment was detected with tonic contractions. Spikes associated with phasic contractions were also recorded by microelectrodes. The threshold for the phasic response is lower than for the tonic at short stimulus durations, and tonic threshold is lower at long durations. At high intensities both responses occur; phasic chronaxie is 10 msec., tonic 70 msec. Velocity of the phasic action potential is 2–3 cm./sec. Time for half-relaxation of maximal phasic contraction is 0.6 second, for tonic 3.0 seconds, for the two together 45.5 seconds. The phasic contractions facilitate markedly and reach maximum tension at 10/second; the tonic show less facilitation and are maximal at 2–3/second. High potassium (100m*M*) eliminates the phasic but leaves the tonic contraction. Omission of Mg^{++} is without effect but low Ca^{++} eliminates both contractions. Procaine and tetracaine are ineffective and the muscle is very responsive to mechanical stimulation. It is concluded that conduction is from muscle fiber to fiber, that the phasic and tonic systems are closely coupled, but that activation of the tonic may occur without membrane action potentials.

On the phosphatide composition of sea anemones. MAURICE M. RAPPORT AND EUGENE L. GOTTFRIED.

It was reported (Bergmann and Landowne, 1958) that the phosphatide composition of the west coast sea anemone, *Anthopleura elegantissima,* differed remarkably from that of the anemone of India, *Gyrostoma* sp., (Rajagopal and Sohonie, 1957). Whereas *Gyrostoma* contained cephalin, lecithin, and sphingomyelin in the proportion 4:12:1, *Anthopleura* contained only sphingomyelin and a choline plasmalogen in the proportion 20:1. The plasmalogen was reported by Bergmann and Landowne to have a cyclic glyceryl acetal structure rather than the novel α,β-unsaturated ether structure of mammalian plasmalogens. In contrast, Rapport and Alonzo (1960) showed that lipids of the east coast sea anemone, *Metridium senile,* contained high concentrations of amino plasmalogen having the unsaturated ether structure. The phosphatides of *A. elegantissima* were therefore reexamined and compared by thin layer chromatography with those of *M. senile*. The findings of Bergmann and Landowne could not be confirmed. *Anthopleura* lipids contained both ethanolamine and choline glycerophosphatides in approximately equal amounts; the quantity of sphingomyelin was quite small. Plasmalogen was associated predominantly with ethanolamine phosphatide (cephalin). Both *Anthopleura* and *Metridium* had compositions of complex lipids that were very similar and in accord with that reported for *Gyrostoma*, except that the quantity of cephalin relative to lecithin was substantially higher. Inasmuch as the methods used to analyze *Gyrostoma* lipids did not take into account the special properties of the plasmalogen component of the ethanolamine phosphatide, it is reasonable to assume that *Anthopleura*, *Metridium*, and *Gyrostoma* have phosphatide compositions that are similar (1) in content of cephalin, lecithin, and sphingomyelin, and (2) in having phosphatidal ethanolamine as the predominant plasmalogen. The error in the studies of Bergmann and Landowne was very likely caused by their use of alcohol-preserved animals. The plasmalogen they found to have a cyclic glyceryl acetal structure was probably an artifact.

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Patterns of chemically induced reversions among mutants of Salmonella typhimurium. J. L. ROSNER.

Freese has made an intensive study of the mutational properties of the rII region of bacteriophage T4. He found that 98% of the mutants induced by 2-aminopurine (2AP) would revert to wild type upon treatment with base analogs. In comparison, 87% of nitrous acid (HNO_2)-induced mutants and 14% of spontaneously arising mutants are induced to revert with base analogs. In an early study with histidineless mutants of *Salmonella typhimurium*, Kirchner reported that 4% of the spontaneous and 25% of the 2AP-induced mutants would revert after treatment with 2AP. Dr. A. Eisenstark and the author have studied the reversion pattern of nearly 200 cysteineless mutants of *Salmonella* (manuscript in preparation). Ninety per cent of the 2AP-induced mutants and about 60% of both spontaneous and HNO_2-induced mutants were found to be revertible with 2AP. In the present study, Kirchner's work was reexamined utilizing a more sensitive assay for 2AP mutagenicity.

For each mutant, *ca.* 10^{-7} cells are spread on minimal plates enriched with 2.5% broth. Diethyl sulfate (DES) is added to a small paper disc on the plate. The mutagens 2AP and 5-bromdeoxyuridine (5BD) are added directly to the plate. If the disc is used, a majority of the positive responses are concealed by the disc. The results of these experiments corroborated the findings with the cysteineless mutants. Of the 11 2AP-induced mutants originally tested by Kirchner, all were found revertible by DES and 2AP. Eight of eleven were also revertible using 5BD. Of the 20 spontaneous mutants tested, two reverted with all three mutagens, 10 with DES and 2AP, and 6 with DES alone. In a preliminary study with 13 histidineless mutants induced by HNO_2, two were revertible with DES, one with 2AP and none with 5BD. Further investigation is planned.

Thus, spontaneous mutations in bacteria and bacteriophage respond differently to reversion by chemical mutagens.

Mechanism of chromatophore control in the common sand flounder Scophthalamus aquosus. GEORGE T. SCOTT, RICHARD L. CLARK AND JAMES C. HICKMAN.

The great majority of teleost fishes investigated reveal evidence of dineuronic control of chromatophores, *i.e.*, the presence of both aggregating and dispersing nerve fibers. In our work on the sand flounder we have found no evidence of dispersing nerve fibers or the necessity to postulate a dispersing neurohumor. The experiments are summarized as follows: (1) Sectioning of the sympathetic chain in a light-adapted fish causes rapid dispersion of chromatophores on the body posterior to the cut; electrical stimulation produces blanching due to concentration of the melanin pigment. (2) Sectioning of spinal nerves, or the application of a pressure block to them, causes dark banding due to melanocyte dispersion. (3) Such a dark band fades within one to two days when the fish are maintained on a light background. (4) Recutting distal to the first cut does not produce a second band within the area of the first.

This kind of operation, in fish where a second band is formed, has been used as evidence of functional dispersing nerve fibers by a number of investigators working on several kinds of teleosts. Chromatophores in the sand flounder disperse when the aggregating nerve fibers are separated from the CNS. Stimulation of these fibers causes concentration of pigment. (5) Acetylcholine or eserine injected in the cord of a dark-adapted fish produces transitory light banding, presumably by cholinergic facilitation. Some twenty other drugs studied produce dark banding, presumably by pharmacologic inhibition. Acetylcholine or eserine have no effect when injected subcutaneously. (6) The following drugs were observed to produce persistent localized lightening when injected subcutaneously: epinephrine, norepinephrine, isopropyl arterenol, serotonin, and the monoamine oxidase inhibitors, pheniprazine and phenelzene. (7) A large number of depressant drugs including tranquilizers and sedatives (except barbiturates, which are inactive) produce localized darkening. The serotonin-blocking agents, lysergic acid diethylamide and bimaleate, also produce darkening as do the epinephrine-blocking agents, phenoxybenzamine and N-2-chloroethyl dibenzylamine. Pituitary extracts are inactive on both normal and denervated chromatophores.

The most active lightening agents are the epinephrines and, on the other hand, epinephrine-blocking agents are potent drugs causing chromatocyte dispersion; therefore, it seems likely that a catechol amine similar to epinephrine is the chemical transmitter of the aggregating nerve fibers.

The study was aided by a National Institute of Mental Health Grant MY-3903 to Oberlin College.

Effect of carbon dioxide on gas exchange in Thyone briareus. David M. Travis.

Sea cucumbers, *Thyone briareus,* were exposed to carbon dioxide pressures (pCO_2 to 40 mm. Hg) in air at atmospheric pressure and the exchange of oxygen and carbon dioxide measured. Respiratory chambers consisting of syringes with rubber caps were loaded with animals, sea water and air containing various concentrations of carbon dioxide and shaken in a water bath at 22° C. Gas samples were taken at one-hour intervals and analyzed volumetrically. Animals were blotted, weighed and their volume determined by weight of displaced sea water. Oxygen uptake and carbon dioxide output were calculated from changes in gas concentrations and volumes. Oxygen tensions were allowed to fall only 15–20 mm. Hg. Initial oxygen concentrations were adjusted to that of air.

Oxygen uptake fell with increasing carbon dioxide tensions. This decrease was 22% less when the pCO_2 was raised from 3 mm. Hg to 12 mm. Hg, 34% at pCO_2 of 25 mm. Hg, and 55% at 40 mm. Hg. There was a slow rise of carbon dioxide output from the animal exposed to air without added carbon dioxide, and a plateau was reached in 4 or 5 hours, then giving respiratory exchange ratios of around 0.75. Sea cucumbers require longer periods for equilibration with higher concentrations of carbon dioxide. Oxygen uptake varied with time in single animals. The pattern of variation was similar in animals studied simultaneously in parallel experiments, whether exposed to carbon dioxide or not.

The decrease in oxygen uptake of *Thyone briareus* on exposure to higher carbon dioxide tensions is similar to that previously found in another sand- and mud-dwelling marine invertebrate, *Golfingia gouldii.* The pCO_2 may be ten- or thirty-fold higher in sand than in sea. The results suggest a possible physiological function of carbon dioxide in the regulation of respiratory metabolism of these animals in the natural surroundings.

Carbon dioxide inhibition of growth and respiration in Tetrahymena. David M. Travis, Alfred M. Elliott and Il Jin Bak.

Tetrahymena pyriformis E, growing in broth, was exposed to air with carbon dioxide tensions varying from 1 to 350 mm. Hg at atmospheric pressure and the changes in respiration and growth measured. Respiratory vessels consisted of 30-ml. syringes and 350-ml. tonometers which were immersed in a water bath at 25° C. Glass capillaries placed in the tonometers permitted recording of pressure changes. The time and volumes of culture and gas were adjusted so that oxygen concentrations were maintained between 21% and 18% during single periods of gas sampling. The gases were analyzed by the ½-cc. method of Scholander. Oxygen uptake and carbon dioxide output were calculated from changes in gas concentrations and the known volumes and pressures of the vessels. Cells were examined by light microscopy to determine the number of dead and living ones. Relative changes in number of organisms were estimated turbidimetrically.

Growth in 24 hours reached a maximum at carbon dioxide tensions below 40 mm. Hg and is progressively less with higher tensions up to 350 mm. Hg. Fifty per cent inhibition of growth occurs at 90 mm. Hg, 90% at 220 mm. Hg and above 95% at 350 mm. Hg. Cells survived for 72 hours in an environment of air with carbon dioxide tensions of 220 mm. Hg but not with 350 mm. Hg. Changes in oxygen uptake and carbon dioxide output paralleled the changes in growth.

Logarithmic growth protozoa washed twice in distilled water (resting cells) and placed in syringes without added buffer for periods of one to two hours showed a decrease in oxygen uptake of 10% when the carbon dioxide tension was increased from 3 to 25 mm. Hg. Similar changes were noted in broth cultures studied over short periods in which there was little change in number of organisms.

PAPERS READ BY TITLE

Correspondence of maximum response of snails to magnetism with the strength of geomagnetism. FRANKLIN H. BARNWELL AND FRANK A. BROWN, JR.

During a two-month period in the summer of 1960 an attempt was made to determine the optimal strength of the horizontal vector of magnetism for effecting orientational changes in the snail, *Nassarius.* The turning response of south-bound snails to an abrupt experimental reversal of the field by a bar magnet at each of eight strengths—0.04, 0.1, 0.2, 0.4, 0.8, 2.0, 5.0, and 10.0 gauss—was assayed. The results indicated the maximum response, left-turning, to lie at 0.2 gauss with decreasing response to both stronger and weaker fields. Two overlapping repetitions of the experiment, a total of three months, were attempted during the summer of 1961. Again the maximum response lay at 0.2 gauss but the response was *right*-turning. A third attempt to resolve the problem in the summer of 1962 resulted in June–July in a striking repetition of the 1960 pattern and in July–August of the inverted pattern. Considering all seven months of data (involving 51,040 snail paths) analysis of variance revealed no significant differences among the means. However, the variances themselves for strengths of the series in increasing order were, respectively, 1.43, 10.03, 10.23, 3.79, 2.79, 3.36, 1.34, and 4.23. Highly significant F ratios indicate a mirror-imaging response with maximum sensitivity straddling, remarkably, the earth's horizontal vector, namely 0.17 gauss. Factors determining response sign remain still obscure. Left-turning response in 1962 was accompanied by return to the same clear monthly variation reported for the effect of fields within a factor of 4 of the earth's F (0.6 gauss) relative to ones greater than 4. The comparable variation during right-turning periods appeared to be semimonthly.

This study was aided by a contract (1228-03) with the Office of Naval Research and by grants from the National Science Foundation (G-15008) and the National Institutes of Health (RG-7405).

Orientational responses in organisms effected by very small alterations in gamma (Cs^{137}) radiation. FRANK A. BROWN, JR., H. MARGUERITE WEBB AND LELAND G. JOHNSON.

Preliminary experiments indicate organismic sensitivity to weak gamma radiation and to direction of the gamma source. The response varies with time and the animal's geographic orientation. Plane gamma sources, four inches in diameter, contained 24 μC. of Cs^{137}. The sources, on outside of apparatus, produced a 6-fold increase in radiation at animals' position. Sources and dummy sources were enclosed in packages; the observers were uninformed as to content. In Woods Hole, snails southbound morning, afternoon and evening were subjected to shuffled experimental series consisting of 6 groups of 10-path samples under three conditions—dummy, gamma to left, gamma to right. July 23–30, inclusive, gamma increase, both directions, effected right-turning mornings, left-turning afternoons, and no mean turning evenings. Analysis of variance yielded $P < .001$. From July 23 through August 24, 1962, analysis of 24,120 paths demonstrated a steady, apparently linear, drift in the difference between morning and afternoon response, with a change of sign occurring about August 15. Superimposed on the drift was, suggestively, a semimonthly variation with minima just before new and full moon. Gamma_R response minus gamma_L response displayed a relatively large amplitude semimonthly variation with maxima just before new and full moon. Experiments in Evanston, Illinois, with 15-path samples of planarians, August 7 through 20, 1962, involving 20 groups of shuffled dummy left, dummy right, gamma right, gamma left, for both North- and West-directed worms disclosed for the former direction that 32 cases showed turning from the source, 7 cases toward it, and 1, no response ($P < .001$). Corresponding figures for west-directed worms were 17, 20, and 3.

This study was aided by a contract (1228-03) with the Office of Naval Research and by grants from the National Science Foundation (G-15008) and National Institutes of Health (RG-7405).

Inductive potencies of the manubrium of Tubularia. Allison L. Burnett and Norma A. Diehl.

Numerous investigators have attempted to interpret form in *Tubularia* by postulating the presence of an inhibitor which diffuses proximally along the stem and suppresses the formation of a hydranth in areas adjacent to the primary or existing hydranth. We have found that a small piece of manubrium (one-sixteenth the size of the original manubrium or 0.1–0.3 mm. in diameter), when grafted to the proximal cut surface of a large, healthy 5-mm. stem piece bearing a hydranth, will induce the formation of a new hydranth within two days. Twenty-three out of 31 animals treated in the foregoing manner formed normal tentacle ridges, while 24 control animals which had simply been excised showed no signs of hydranth formation whatsoever.

Within three to five hours after application of the manubrial portion, the coenosarc projects at least 1 mm. beyond the excised perisarc. Once this shift has occurred, the formation of a perfectly developed hydranth is invariably the outcome. Without manubrial induction, the coenosarc retreats back inside the perisarc and remains in this position for several days.

The grafted manubrium is not contributing directly to hydranth formation, but is furnishing a diffusible factor which stimulates adjacent tissue to initiate hydranth formation. If manubrial portions from *Tubularia crocea* are grafted to another species of *Tubularia* (unclassified at the present time), the *crocea* fragment induces a hydranth characteristic of the unclassified species within two days. Grafted stem tissue lacks these inductive potencies.

We feel that it is necessary to interpret form in *Tubularia* by considering the presence of a diffusible growth-stimulating factor in the manubrium, and not rely solely for an interpretation upon the presence of an inhibitor(s) which diffuses proximally along the stem.

The relation between inductive regions and interstitial cell distribution in Hydra pirardi, Tubularia crocea, and Hydractinia sp. Allison L. Burnett, Norma A. Diehl and Ellen Mutterperl.

In the previous abstract it was stated that the manubrium of *Tubularia* contains an inductive principle which is capable of diffusing into the tissues of another species of the same genus, and initiating hydranth formation. Similar observations have been reported for the common hydra, and recently it was demonstrated that the manubrial regions of feeding polyps and gonozooids of *Hydractinia* also possess inductive capabilities.

Toluidine blue-stained whole mounts of these three forms have revealed that interstitial cell distribution is intimately linked with inductive areas in the following manner. (1) The manubrium or primary inductive area contains few interstitial cells. (2) Areas immediately adjacent to the inductive area contain dense concentrations of interstitial cells. (3) Proximal to this region is the so-called gastric region which contains about half as many interstitial cells per unit area as that observed in sub-manubrial areas. (4) The gastric region is invariably followed by another region of high interstitial cell population. In hydra this area is the budding area, in *Tubularia* it is the region where the gonophore stalks and proximal tentacles arise, in *Hydractinia* it is the point from which a stolon begins to grow.

It is postulated that in all three species an inductive principle diffuses proximally from the manubrium, stimulating interstitial cells and probably other cell types to divide repeatedly, thus creating a zone of growth. This growth results in the production of inhibitors which diffuse proximally to the gastric region, suppressing growth activities. The inhibitors invariably become dilute or rendered impotent at a precise level along the gastric region, and at this level a second growth region is initiated.

Neural activity during hypoxia in adult firefly. ALBERT D. CARLSON.

Anoxia in the adult firefly, *Photuris pennsylvanica*, inhibits flashing and may elicit a dull, structureless, hypoxic glow. If oxygen is readmitted after the onset of the hypoxic glow a brilliant flash, the pseudoflash, occurs, with a duration of approximately 5 times that of the spontaneous flash. Neural activity in the lantern was monitored to determine whether the hypoxic glow is initiated by neural activity or by anoxia acting directly on the photocytes or tracheal end cells.

Tank nitrogen was led into a transparent chamber holding the firefly, ventral side up. Platinum recording electrodes, penetrating a small opening in the chamber top, were placed in the photogenic tissue of the sixth abdominal segment. The neural activity was led into a Grass P5 A.C. preamplifier, displayed on one trace of a Tektronix 502 oscilloscope, and stored on tape. The light response was recorded using a photomultiplier, the output of which was frequency modulated for storage on the other channel of the tape.

If neural bursts occurred early in hypoxia an hypoxic glow was initiated which increased stepwise with each burst. Neural bursts were usually not observed during later hypoxia but a gradual increase in glow intensity accompanied the onset of random neural and muscular activity. Those parts of the organ glowing in air produced an hypoxic glow apparently without neural stimulation.

It appears that the hypoxic glow is the result of neural activity during hypoxia which initiates the formation of light producing complex. These results can explain why the actively flashing firefly will readily produce an hypoxic glow and pseudoflash while the quiescent, non-flashing animal will not.

Protein changes in the ageing lobster. ALFRED B. CHAET AND DAVID BAU, JR.

Since previous studies have shown a fundamental difference between the blood proteins of young and old horseshoe crabs, it seemed advantageous to analyze the blood proteins of the developing lobster, *Homarus americanus*. In the present study both starch gel and disc electrophoretic techniques were used. In some experiments, blood was removed by cardiac puncture, whereas in other instances repetitive freezing-thawing released adequate fluid for electrophoretic assay. Lobsters representing seven different age groups, ranging from the developing lobster still in its egg case to the five-year-old animal, were studied. The approximate age of the various lobsters analyzed was as follows: seven-day pre-hatched, one-day post-hatched, three-day, seven-day, fourteen-day, two-year and five-year.

The electrophoretic patterns obtained from representative samples of the various groups differ with age in that only two protein bands found in the younger forms were still present in the oldest animals. Three or four protein bands disappeared as the animal ages, whereas six or eight proteins, which were not found in the younger forms, made their appearance in the two- and five-year-olds. Except for one protein band (egg protein) found in the youngest form, the disappearance of proteins in the various age groups was a gradual rather than sudden phenomenon. Similarly the appearance of three new protein bands occurred gradually with age.

Although previous studies have shown a gamma globulin-like protein in the blood of the young horseshoe crab, which was absent in the older form, no gamma globulin-like protein bands were found by either electrophoretic techniques in any of the seven lobster age groups studied.

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Gas absorption from fish swimbladder. C. Lloyd Claff, with the technical assistance of Arthur Richmond and Gladys Harrison.

A hypodermic needle, the hub of which was fitted with a vaccine plug, was introduced into the swimbladder, and sutured in place. All gas was removed by needle and syringe through the vaccine plug, volume noted, and a sample analyzed for CO_2; O_2; and N_2. A volume of gas, either CO_2; O_2; N_2; He; or Ar, equal in volume to the gas removed, was introduced into the swimbladder. Samples of gas were withdrawn at 30 seconds; 1 minute; then every minute to 5 minutes; then at 10-minute intervals to 30 minutes; again at 1 hour; 2 hours; 3 hours; and 4 hours. Samples of venous blood were removed as soon thereafter as possible after each of the above intervals. Each gas sample was analyzed for CO_2; O_2; and N_2. Each sample of blood was analyzed for extracted CO_2 and O_2; and the pH of each sample was taken.

Absorption of CO_2 was remarkably fast. Ninety per cent of the CO_2 from the swimbladder lost in first 10 minutes was replaced by O_2 and N_2. Within six minutes CO_2 in blood was between 32 and 58 volume per cent. (All values not corrected for S.T.P.D.) The pH of venous blood dropped to 6.8 and stayed at that value for 40 minutes. Within 10 minutes O_2 and N_2 volume per cent values in swimbladder gas returned to normal values for atmospheric pressure.

Fish survived 24 hours after the experiment. The experimental fish were northern porgy (scup), weighing one to one and a half pounds.

Data on other gases will be reported at a later date.

Effects of KI on G–ATP and G–ADP actin. Eloise E. Clark and Richard F. Olivo.

Both G–ATP and G–ADP actin can, under appropriate conditions, polymerize to form a high viscosity product. Since the polymerization of the former is accompanied by dephosphorylation of the ATP, while that of the latter is not, it is of interest to know whether the mechanism of polymerization and the resulting F-actins are the same. An approach to such an analysis is feasible through the use of KI. Preliminary findings indicate that the processes (and products) may be distinguishable.

Szent-Györgyi and Szentkiralyi have shown that G–ADP formed by the depolymerization of F-actin by high concentrations of KI does not repolymerize when the ionic strength of the solution is lowered unless an ATP system is present. Hayashi and Rosenbluth have shown that when F-actin is depolymerized in water containing Mg^{++}, the resulting G-ADP can be repolymerized at 29° C. by the addition of .1 *M* KCl. The present experiments show that when KI is added to the water-depolymerized F-actin, repolymerization of the G–ADP does not occur; further, a sample of G–ADP in KI was found to have a sedimentation coefficient of 3.12×10^{-13} S. In addition it has been found that when KI is added to G–ATP solutions at 0° C., both the rate and extent of polymerization are markedly lowered. This is in contrast to the polymerization of G–ATP at 29° C. where both KCl and KI are almost equally effective in producing high viscosity products.

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Influence of brain lesions on melanocyte dispersion. Richard L. Clark and George T. Scott.

It has been firmly established that melanocyte stimulating hormone (MSH), secreted by the pars intermedialis, is the darkening hormone in *Rana pipiens*. An attempt was made to elucidate experimentally the neural mechanism by which the pituitary's secretion of MSH is controlled.

Various semi-micro neuro-surgical techniques were employed systematically to ablate regions in the brain. The most successful operative procedure was our use of a micro-chemical cautery, consisting of bichloracetic acid injected (0.25–0.50 mm.^{3}) by means of a drawn out glass micropipette (0.1 mm. in tip diameter).

Frogs with discrete lesions in the posterior-ventral wall of the diencephalon showed spontaneous and persistent melanocyte dispersion. Animals similarly treated but in different areas (cerebellum, cerebrum, optic lobes, etc.) showed only a transient post-operative darkening together with expected behavioral changes. It is probable that a pituitary-mediating suppressor area exists in the diencephalon, and the removal of this neuro-secretory control center results in spontaneous hyperactivity in the intermediate lobe.

Recent investigations in our laboratory have shown that many of the clinically potent tranquilizers (the phenothiazines, reserpine and meprobamate) produce melanocyte dispersion after intraperitoneal injection. Evidence abounds in the literature concerning the subcortical target areas in higher vertebrates for tranquilizers. Our work suggests that there is located in the frog diencephalon homologous target nuclei which control the pituitary's secretion of MSH.

This study was aided by a National Institute of Mental Health grant MY-3903 to Oberlin College.

Contraction of the epidermis during tail resorption in the ascidian Amaroucium constellatum. RICHARD A. CLONEY.

The larval notochord of *Amaroucium* is bound by an extracellular membranous sheath. Squamous notochordal cells form a second membrane within the sheath and the axis of the notochord is filled with a clear extracellular matrix. The muscle cells of the tail are attached at their inner surfaces to the sheath. The nerve cord lies adjacent to the notochord on the left side of the tail. These tail tissues are closely bound by a thick squamous epithelium comprising the epidermis. Two layers of tunic are disposed outside the epidermis. The outer layer is lost at metamorphosis; the inner layer is retained.

Tail resorption is completed within 6 to 8 minutes after the beginning of metamorphosis. Early changes are detectable in the notochord. The matrix rapidly disappears, probably passing into the trunk, and the notochord-muscle-nerve cord (NMN) complex partially collapses. Simultaneously, the epidermis lifts away from the underlying NMN complex. A fluid-filled space is formed between these components. The NMN complex buckles and begins to move into the posterior region of the trunk. The epidermis appears to be under tension at this time. At the end of tail resorption the epidermis forms a thick cap at the posterior end of the trunk, enclosing the folded NMN complex.

If the epidermis is ruptured, or if the tail is excised after tail resorption begins, the epidermis in *both the anterior and posterior fragments or halves of the tail shorten independently,* forming compact ring-shaped masses at the base and tip of the tail, respectively. Under these circumstances the NMN complex is never withdrawn and never shortens by itself. After tail resorption begins it can be reversibly inhibited with 10^{-2} *M* KCN (in sea water at pH 8.0). These observations support the hypothesis (Cloney, 1961) that contraction of the epidermis is the major motive force involved in ascidian tail resorption.

Fine structure of acrosome and early fertilization stages in Saccoglossus kowalevskii (Enteropneusta). ARTHUR L. COLWIN AND LAURA HUNTER COLWIN.

The acrosome is a membrane-bounded vesicle with a single shallow tubular invagination in the region adjoining the nucleus. Within this vesicle a large acrosomal granule adjoins the invagination but does not extend as far as the acrosomal apex. A layer of fine granular material lies between the acrosomal granule and the acrosomal membrane, except at the apex. Dense material widely separates the acrosomal membrane from the plasma membrane except at the apex, where the two membranes lie very close together. Two envelopes surround the fertilizable egg but they are not penetrated by egg microvilli.

The early stages of fertilization are as follows. The tip of the spermatozoon attaches to the outermost envelope; the acrosome opens or dehisces apically. Around the rim of dehiscence the acrosomal and sperm plasma membranes are then seen to be fused, constituting one continuous unit sperm plasma membrane. Then the shallow acrosomal invagination lengthens into a long acrosomal tubule (formerly called "filament"), while the acrosomal vesicle everts. The large acrosomal granule disappears.

The acrosomal tubule penetrates the egg envelopes, fuses with the egg plasma membrane, and opens apically. Thus the zygote plasma membrane is formed; although it is clearly one continuous unit membrane, it arises, then, as a mosaic of the sperm and egg plasma membranes. The sperm nucleus, mitochondria, etc., pass through the acrosomal tubule and enter the fertilization cone. During this passage the nucleus elongates greatly as though squeezing or being squeezed through the tubule.

The acrosomal structure and the main events of sperm-egg association are basically the same as those in the annelid *Hydroides* (Colwin and Colwin, 1961). Since the same pattern obtains in species of two such divergent phyla, it is conjectured that this pattern is a fundamental one, which will be found to occur widely.

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Induction of spawning in Saccoglossus kowalevskii (Enteropneusta) at Woods Hole. LAURA HUNTER COLWIN AND ARTHUR L. COLWIN.

Animals spawned occasionally when one or more were kept in bowls of sand in running sea water, but a routine method was desired for obtaining gametes predictably at convenient times. Developing embryos collected in the field were compared with timed, artificially inseminated, eggs; natural spawning seemed to occur usually in the middle of the night. Nevertheless, experimental darkening or various changes in the lighting cycle induced no shedding. Then, since the temperature of the natural habitat rose considerably during low tide on sunny days, experimental variations in water temperature were investigated. Warming was found to induce shedding; the eggs, however, were in best condition when kept cool.

The following procedure finally evolved. Clean animals, in separate bowls of filtered sea water are kept at *ca.* 27° C. for 7 to 8 hours, then placed in cool filtered sea water (*ca.* 22° C.) and cleaned repeatedly to remove the mucus they secrete. For years this method has succeeded with some 90% of specimens judged capable of spawning (over 1000 animals). Some animals spawned while warm. But 70% spawned after cooling, more than half beginning by 10 to 120 minutes, the rest, up to six hours or more. Gametes can be obtained at any desired time of day.

Animals are most suitable for use when healthy, with no unhealed wounds. Much of the proboscis, the entire collar, and some of the genital region must be intact. With reflected light, the usually blue genital regions of ripe females show large discrete eggs through transparent body walls; male genital regions are ripe when distended and creamy or peach, but not brown, in color.

Small samples of normal spermatozoa can conveniently be obtained by biopsy of non-heat-treated males but, thus far, comparable biopsy of untreated females has not yielded eggs which subsequently could be fertilized.

This study was aided by Research Grant RG-4948 from the National Institutes of Health.

Observations on the gas-secreting epithelium of Physalia. EUGENE COPELAND.

Observations on *Physalia* collected in the Woods Hole area were made by use of the electron microscope. The gas gland of *Physalia* is composed of three layers, ectodermal, mesogleal and entodermal (gastrodermal). The ectodermal layer faces the gas cavity and presumably secretes the specific gas, carbon monoxide (Wittenberg). In osmic-fixed material the distal ends of the ectodermal layer of cells reveal complicated gatherings of cisternae leading to the free surface. Orderly rows of vesicles extend from the tips of the cisternae. The bodies of the cells show a homogeneous cytoplasm with a reticular arrangement of small nondescript granules. The nuclei are basal and surrounded by a satellite of small vesicles. Just distal to the nucleus there is a layer of multivesiculate bodies. At the level of the nucleus, the cytoplasm becomes quite dense and can be easily traced into the projections which penetrate the mesoglea and form complicated indigitations with projections from the entoderm cells. Permanganate-fixed material shows, in some cases, double membrane profiles in the distal part of the cell (where osmic fixation revealed only reticular cytoplasm). There were also indications of Golgi-type profiles in the multivesiculate layer. It is now interpreted that the fixations are reasonably good and the variable picture is ascribed to possible degenerative stages of *Physalia* which have traveled a long distance in the Gulf current and, after being blown out of that current into the cold waters off Martha's Vineyard, are near their end. It is planned to check this by study of animals in the Gulf itself or with more predictable collecting in the Gulf stream off Florida.

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Compartmentalization of chloride in lobster muscle. Philip B. Dunham and Harold Gainer.

Chloride, potassium, and sodium concentrations in the walking leg bender muscle of *Homarus americanus* were determined after treatment under various ionic conditions at 5–7° C. For muscles in normal medium (Na_o, 455 mM; K_o, 15 mM; Cl_o, 534 mM), ion concentrations, determined by elemental analysis and expressed as mM/kg. wet weight of cells (corrected for extracellular space, determined using C^{14}-inulin) were: K_i, 122 mM/kg.; Na_i, 84 mM/kg.; Cl_i, 80 mM/kg.

Results from three kinds of experiments suggested that intracellular Cl is situated in two distinct compartments. (1) The kinetics of net efflux of Cl_i were followed for 24 hours from muscles placed in 15 mM Cl_o medium. The efflux was nearly complete at 14 hours, at which time Cl_i was 30 mM/kg. (2) Muscles were equilibrated for 24 hours in media with constant K_o and changing Cl_o (534–15 mM). Cl_i varied linearly with Cl_o over this range at two different concentrations of K_o (15 and 45 mM). The slope and extrapolation to zero Cl_o was the same at both K_o's. The value at zero Cl_o was about 30 mM/kg. In both cases, K_i and Na_i were constant at all levels of Cl_o.. With 45 mM K_o, Na_i and Cl_i were equal to that obtained in 15 mM K_o, whereas K_i was 145 mM/kg. (3) The rate and extent of exchange of Cl_i with trace Cl^{36} added to the medium were determined for a 24-hour period. In 534 mM/Cl_o there was no further exchange after 14 hours, at which time 65% of Cl_i had exchanged, leaving 30 mM/kg. not exchangeable. Cl^{36} was added to the medium of muscles after 6 hours in 15 mM Cl_o. There was no further exchange after 7 hours, at which time only 5% of Cl_i was exchanged, again leaving 30 mM/kg. not exchangeable.

That there is a compartment of immobile Cl_i, 30 mM/kg. is indicated by two independent methods. This compartment is constant over a wide range of Cl_o, and is not exchangeable with Cl_o. There is also a compartment of mobile Cl_i in equilibrium with Cl_o, with a constant Cl_o:Cl_i ratio of about 10:1 over a wide range of Cl_o.

Two physiological varieties of Noctiluca miliaris. Roger Eckert and Margaret Findlay.

Noctiluca is widely familiar because of its role in the occurrence of marine bioluminescence and red tides. In spite of its almost world-wide distribution, only one species, *Noctiluca miliaris* (synon. with *N. scintillans*), has been recognized. However, Sweeney (personal communication) noted that *Noctiluca* collected in the Pacific off San Diego did not luminesce and were smaller than a luminescent variety collected in the Gulf of California.

To extend these observations we are culturing *Noctiluca* collected from both the North Sea and from Puget Sound. The North Sea cells luminesce and range in diameter from 400 μ to 850 μ, whereas the Puget Sound cells are non-luminescent and range from 200 μ to 450 μ. Although no other morphological differences have been noted, work is in progress to examine possible ultrastructural differences between the luminescent and non-luminescent varieties.

The bioelectric and bioluminescent behavior of both types of *Noctiluca* were investigated as described elsewhere (Eckert, this issue). Both types respond at similar stimulus current intensities with an all-or-none negative-going action potential as large as 70 mv. This potential is followed by a distinct movement of the tentacle. In the North Sea culture the action potential is also invariably followed by a flash of luminescence. On the other hand, even with multiplier sensitivities three orders of magnitude greater, no light emission could be detected in any of the Puget Sound cells.

In addition to the all-or-none evoked potentials, slow, spontaneous rhythmic potentials were routinely recorded from the luminescent variety. These potentials are negative-going and of a magnitude similar to the evoked potentials; however, they are not all-or-none, are 20–40 times as long as the evoked spikes, and are never accompanied by luminescence. Spontaneous potentials were never recorded in the nonluminescent type.

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Survival of Tetrahymena at elevated oxygen pressures. Alfred M. Elliott, David M. Travis and Il Jin Bak.

Tetrahymena pyriformis E, grown axenically in broth cultures, was subjected to 100% oxygen under varying pressures from 1 to 2.5 atmospheres. Plastic 10-ml. syringes were employed as pressure chambers in which glass capillaries were placed to register pressure. The syringes were maintained at room temperatures (25–27° C.) in a horizontal position to afford maximal exposure of the cells to the gas. Initial and final numbers of cells were recorded turbidimetrically. The cultures were examined with the light microscope to determine numbers of living and dead cells. Hydrogen ion changes during the experiments were noted and the O_2 and CO_2 analyses were done with the ½-ml. Scholander technique.

Protozoa exposed initially to 2.5 atmospheres of 100% oxygen show a linear increase in death of the cells with time. Ten per cent are dead at 6 hours, 25% at 9 hours, and over 90% at 12 hours. At one atmosphere, 100% oxygen is less toxic. Only 10% of the cells are dead in 9 hours and 25% at 12 hours. During the first 3 hours of exposure at both 1 and 2.5 atmospheres of 100% oxygen cell division appears normal, but thereafter mitosis ceases and the cells begin to die, preceded by characteristic swelling to form spheres. At 2.5 atmospheres of oxygen the CO_2 output is approximately twice that at 1 atmosphere. The O_2 uptake rises to a peak in 6 hours at 2.5 atmospheres. At 1 atmosphere the peak appears at 9 hours, thereafter declining. The pH falls from 6.9 to 6.7 during the 12 hours of exposure at both 1 and 2.5 atmospheres.

Oxygen pressure dose response demonstrates a straight line relationship with CO_2 output. Oxygen uptake declines uniformly with increasing oxygen pressure.

Effect of phenothiazine derivatives on the permeability of the dogfish erythrocyte. Alan R. Freeman and Morris A. Spirtes.

Three phenothiazine tranquilizers, varying in clinical potency, were tested for their ability to reduce hemolysis when erythrocytes were exposed to hypotonic, partially hemolytic saline solutions. Trifluoperazine, clinically the most potent tranquilizer, reduced the control hemolysis of 40% to 26% at a final concentration of 2.5×10^{-6} M. Chlorpromazine, less active clinically, produced the same degree of protection at 1×10^{-5} M, and chlorpromazine sulfoxide, a pharmacologically almost inactive compound, had no effect at concentrations up to 1×10^{-4} M. Similarly, chlorpromazine could prevent the swelling of dogfish red cells exposed to hypotonic, non-hemolytic sodium chloride solutions. Spectrophotometrically, chlorpromazine-treated cells also appeared to have a smaller average cell volume, even when suspended in hypertonic salt solutions. Since data using the hypotonic, non-hemolytic technique were obtained by a spectrophotometric method, further experiments will have to be performed before any definite conclusions can be drawn from them.

Absorption spectra from 300 to 700 mμ of dogfish hemoglobin and cyanmethemoglobin showed peaks identical with those of mammalian hemoglobin and cyanmethemoglobin.

The intracellular, ionic pattern of the erythrocytes was determined in eight dogfish. Values of 11.9 ± 4.0 and 101.9 ± 8.7 were noted for sodium and potassium, respectively, expressed as milliequivalents of the ion per 3 millimoles of hemoglobin, using mammalian hemoglobin as the standard in the analyses.

Preliminary experiments indicated that little ion exchange took place in non-hemolytic hypotonic saline solutions. Furthermore, 1×10^{-5} M chlorpromazine did not change the electrolyte balance in these experiments. It thus appears that the phenothiazines primarily affect the movement of water in the various systems tested.

Intravenous injection of 5 mg. of chlorpromazine into a dogfish two feet in length exerted a powerful tranquilizing effect.

In conclusion, evidence has been obtained demonstrating that phenothiazine tranquilizers affect the osmotic movement of water in dogfish erythrocytes to a degree directly related to clinical potencies. This action appears not to be related to ionic phenomena.

Effects of isolation and denervation of crayfish muscle fibers on their membrane resistance. LUCIEN GIRARDIER, JOHN P. REUBEN AND HARRY GRUNDFEST.

Currents were applied with one intracellular microelectrode to muscle fibers of crayfish. Resulting changes in membrane potential were recorded with another electrode. The slope of the current-voltage curve through the resting potential gives effective resistance, which in fibers of neurally intact muscles ranged between 7.7 and $29.6 \cdot 10^4$ ohms. The length constant (λ), measured from the exponential decay of the potential along the fiber, showed much less variation, its value ranging between 2.73 and 3.95 mm. From these respective values for each fiber and from its diameter, the specific resistances of the cell membrane and sarcoplasm were calculated, using the cable equations. There was no significant correlation between diameter and length constant or sarcoplasmic resistance. A positive correlation was found between the diameter and membrane resistance ($r = 0.90$; $P < 0.05$, $n = 7$). This finding suggests the existence of low resistance pathways in parallel with the cell membrane. If they were in essentially radial distribution, their lengths and resistance would increase with the fiber diameter. Structures with these properties have been observed and characterized with the aid of electron microscopy. They are tubules which run inward from the periphery in proximity to the Z lines, and are probably homologs of the T-system component of the triad. They are not connected with the sarcoplasmic reticulum.

In muscles that had been denervated for 7 to 19 days the effective resistance of the fibers did not differ significantly from that of the control muscles of the same animals. The length constant did decrease significantly with a mean of 2.19 ± 0.16 mm. as compared with 3.41 ± 0.41 mm. in the control preparations. The drop is attributable to a decrease of the membrane resistance in the fibers of the denervated muscles to about half the value in the controls: *e.g.*, 3200 ohm cm.^{2} as against 6700 ohm cm.^{2} in fibers of 180 μ diameter.

Isolated single fibers have a still smaller length constant, 1.52 ± 0.02 mm. This indicates a membrane resistance of 1,600 ohm cm.^{2} in a 180 μ fiber. The lower membrane resistances in the denervated and isolated fibers are correlated with correspondingly higher rates of movement of KCl across the membrane for a given driving force.

Studies on the isolated islet tissue of toadfish: the uptake of injected C^{14}-glucose by islet and other tissues. FREDERICK C. GOETZ AND S. J. COOPERSTEIN.

As part of a study of the influence of blood glucose on the secretion of insulin by islet tissue, we have determined the C^{14} content of islet and other tissues of the toadfish following injection of C^{14}-glucose into a gill arch vessel. H^3-mannitol was injected simultaneously as a measure of the extracellular compartment. The samples were counted using the liquid scintillation spectrometer.

Within five minutes after the injection of C^{14}-glucose, the C^{14}-content of islet reached that of blood; this is more rapid equilibration than in any tissue except heart. By 60 minutes the C^{14} content of islet was greater than that of any other tissue except brain. The amount of H^3-mannitol in brain was only about one-fifth that in most other tissues, indicating that brain has a very small extracellular compartment in equilibrium with blood.

The amount of C^{14} found in heart, liver, kidney and gill following the injection of C^{14}-glucose was about the same as that following the injection of C^{14}-urea. This suggests that glucose, like urea, is freely diffusible into these cells. In muscle, there appears to be a barrier to glucose entry; the C^{14} content following C^{14}-glucose injection was only one-fourth that found following injection of C^{14}-urea. Brain seems to have an effective mechanism for concentrating glucose; the C^{14} content after glucose injection was four times that after injection of C^{14}-urea. Islet may also concentrate glucose, since in islet this ratio was 1.6.

When unlabeled glucose was injected to increase the blood sugar level to 350 mg.%

(compared with 25 mg.% for the control) the uptake of glucose was increased proportionate to the blood sugar level in all tissues except brain. This suggests that the glucose uptake mechanism in brain was saturated at a lower blood sugar level.

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Strontium utilization by Arbacia punctulata. II. LIONEL S. GOLDRING, HENRY I. HIRSHFIELD AND IRENE P. GOLDRING.

In order to study the discrimination of marine organisms for calcium over strontium, we have studied the atomic Ca/Sr ratio of *Arbacia punctulata* larvae as a function of the Ca/Sr ratio in the growth medium. As a necessary precursor to this we have studied the development of pluteal larvae in a variety of saline solutions of sea water ionic strength.

Because of limits imposed by solubility considerations, it was necessary to reduce both sulfate and calcium concentrations to explore the maximum range of Ca/Sr ratios. At one-tenth sea water sulfate, plutei with normal or near-normal skeletons were obtained with a calcium concentration of one-half sea water and with Sr at 10 to 30 times sea water concentration. With one-tenth sea water calcium as well as sulfate, very abnormal plutei were obtained with little or no deposition of skeletal material, regardless of the Sr content. These preliminary experiments define limits that will permit us to explore Ca/Sr ratios from 125 to 2.0. Higher ratios can be easily obtained. Lower ratios may be obtained when solubility limits can be explored in greater detail.

Preliminary attempts to determine the weight of skeletal material deposited showed that mass cultures were necessary to obtain sufficient material for chemical analysis. As an alternative to this, radiochemical methods were explored to determine the deposition of both Ca and Sr in the skeleton. At a Ca/Sr ratio of 125, 0.01 μC. Ca^{45} per ml. and 0.10 μC. Sr^{89} per ml. gave measurable activity in isolated skeletons obtained from a 20-ml. culture.

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Incorporation of C^{14}-thymidine into pool and DNA of deuterated sea urchin eggs. PAUL R. GROSS AND GILLES H. COUSINEAU.

The report by Gross and Harding (1961) of DNA synthesis blockade by heavy water in sea urchin eggs has been followed by several descriptions of similar phenomena in mammalian cells. This is in contrast to the well-established ability of many microorganisms to adapt to growth and division in D_2O. The possibility remained that this difference, at least for the invertebrate egg, could be accounted for by a reduced permeability of the deuterated cell to tracer thymidine. A test of this possibility has been made as follows: eggs were fertilized normally and divided into two batches. One was transferred to reconstituted normal sea water and the other to heavy sea water ($D = 85\%$) at ten minutes post-fertilization. These media contained 0.2 μC./ml. of thymidine-2-^{14}C (25 mC./millimole). When the control cells were at the second cleavage (50%), the deuterated cells had not divided at all. The suspensions were each now divided. One half was centrifuged, and the eggs washed quickly but thoroughly with ordinary filtered sea water. The eggs were then pipetted into detergent-treated planchets and dried to a thin film for counting of total label taken up. The remainder of each sample was treated with an equal volume of 10% TCA containing a 500-fold excess of unlabelled thymidine, stored in the cold overnight, and the eggs collected on Millipore filters with 5 μ pores. The filters were presoaked in the TCA-thymidine solution. After washing with 5% TCA and water, the filters were dried and mounted on planchets for counting of the label incorporated into DNA. Counts were made with a thin-end-window Geiger counter system giving a background of approximately 3 cpm., and each sample was allowed to accumulate 6400 counts. The result is that the pool radioactivity in the deuterated cells is as high as, or higher than, that in the controls. Incorporation of label into DNA was, however, strongly inhibited for the deuterated eggs, sufficiently so to account easily for the "blockade" observed in the autoradiograms. Thus, the inhibition of DNA synthesis by heavy water is exerted at the level of nucleoside incorporation or beyond, and the difference between higher cells and microorganisms in this regard acquires some interest.

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Electrophysiological concomitants of the shadow reflex in certain barnacles. G. F. GWILLIAM.

Some electrophysiological concomitants of the shadow reflex in the pedunculate barnacles *Mitella polymerus* and *Lepas anatifera* and in the sessile barnacle *Balanus eburneus* have been investigated. In both pedunculate forms motor output from the supraesophageal ganglion, recorded externally from stalk nerves and circumesophageal connectives during controlled shading of the internally located ocelli, is sharply increased at "off" and is directly related to the degree of shading (controlled with neutral density filters). There is no indication at these recording sites of the "on" stimulus. The casting of multiple shadows of subthreshold duration in *Mitella* does not lead to a "shadow" response in the motor nerves, nor will as many as 30 rapidly applied threshold shadows completely adapt the response. In *Lepas* adaptation occurs after three to six threshold shadows similarly applied. Recordings from the ocellar nerve in *Lepas*, however, indicate that adaptation is a central phenomenon, for the electroretinogram is undiminished for at least ten threshold shadows. Cutting the ocellar nerve or destroying the eye abolishes these responses. Sectioning one of the pair of ocellar nerves in *Lepas* leads to a diminished response in both circumesophageal connectives, but less so in the contralateral than the homolateral connective.

External recording from the ocellar nerves in *Lepas* (second order sensory fibers) and *Balanus* (presumably primary fibers) results in an electroretinogram of simple form with a relatively large negative wave at "on" and a much smaller positive wave at "off" when recorded with one electrode on the nerve and the other in the surrounding sea water medium. As yet no action potentials have been recorded from the ocellar nerve.

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Light-induced pigment migration in the squid retina. W. A. HAGINS AND P. A. LIEBMAN.

Migration of black screening pigments in and around photoreceptors in response to light has often been thought to contribute to light- and dark-adaptation of the retina in arthropods and lower vertebrates. J. Z. Young has shown histological evidence for the same process in the eyes of live octopus. Movement of screening pigment has now been observed directly in isolated slices of living squid retina by infra-red microscopy. In dark-adapted retina, the black pigment lies concentrated in a thin layer bisecting the photoreceptors at the junction of their inner and outer segments. After a flash of orange light, sufficient to activate 10–50% of the photopigment, the black pigment layer divides into a thin part which remains fixed and a diffuse wide band which advances into the layer of outer segments almost to the internal limiting membrane. At 10° C., the migration begins in two minutes, reaches its maximum extent in 20 minutes and recedes in about two hours. At 2° C., migration was not seen after illumination, but on subsequent warming to 15° C., it occurred without further exposure. Incubation of the slices in sodium-free water sea water (Na^+ replaced by $choline^+$) in which the retinal receptor current is reversibly abolished prevents the pigment migration, even if the tissue is returned to a normal sodium sea water immediately after illumination. It is suggested that the pigment response is a local reaction of the photoreceptors to light, depending upon a change in ionic composition of the cells. When the pigment migrates into the outer segments, its effect on the function of the retina is probably two-fold. First, it should reduce the overall light-sensitivity of the photoreceptors by simple shielding. Second, it should markedly restrict the solid angle from which light entering the pupil can reach the photoreceptors, thus producing a sort of Stiles-Crawford effect. This latter action may be very important in the squid retina, since its layer of outer segments is so thick ($\sim 250\ \mu$) and its pupil is so large (f 3.5) that the resolution of its receptor mosaic is basically poor despite its receptors being only 4–6 μ in cross-section. Pigment migration into the layer of outer segments, however, may improve resolution of the retina by nearly an order of magnitude.

The preparation of sea bass lens epithelial whole-mounts for tritium autoradiography. C. V. HARDING, M. B. NEWMAN, F. E. JONES AND H. ROTHSTEIN.

Whole-mounts of the entire layer of rabbit lens epithelium (a single layer of cells) can be prepared for tritium autoradiography. Such preparations have proven useful in localizing the sites of incorporation of tritium-labeled thymidine in normal and injured lens epithelium. It has been found that a small mechanical injury can induce a large number of the surrounding cells to undergo thymidine incorporation and division. Attempts have been made to extend this study to cold-blooded animals. Injury-induced activation of thymidine incorporation has been demonstrated in the lens epithelium of the sea bass, using the whole-mount technique. However, the required exposure time was very long, and the epithelial cells were obscured by a coating of unidentified material (perhaps fragments of lens fibers). Attempts have been made to make preparations without this coating of material, and the following procedure has proven effective: (1) Inject eye with 0.2 ml. teleost Ringer (Forster and Taggert), containing 5 μC. tritiated thymidine, 3 C./mM spec. act. (2) Two to four hours after injection, fix whole eye for 24 hours in Carnoy's solution (3 parts absolute alcohol:1 part glacial acetic acid). (3) Maintain in 70% alcohol for at least 24 hours. (4) Make whole-mount of lens epithelium (*Arch. Ophthalmol.*, 63, 1960). (5) Treat whole-mount for 6 minutes at room temperature in 0.005% crystalline (salt-free) trypsin made up in teleost Ringer. (6) Wash four times in teleost Ringer, once in distilled water, and dehydrate. (7) Film with Kodak AR-10 stripping film, develop at the end of one week's exposure and stain with Harris' hematoxylin. In such preparations, the cell nuclei were well stained, and radioactive nuclei were evident. In control preparations, treated in identical fashion except for the absence of the exposure to trypsin, the nuclei were obscured, and radioactive nuclei were not evident after one week's exposure.

Electron microscopy of the sea gull adrenal. GLADYS HARRISON.

Structures similar to the annulate lamella found in the clam and the snail oocyte by Rebhun have been observed in the adrenal gland of the sea gull. The lamellae in some instances are in intimate association with the nuclear membrane, lending support to Swift's theory of the nuclear membrane being a "mold" on which the lamellae form. When the lamellae are not closely associated with the nuclear envelope, they may assume a variety of patterns, from straight parallel arrays to circular configurations. Vesicles have been observed to be continuous with the ends of the lamellae much in the same manner that the vesicles and membranes of the Golgi apparatus appear.

Annuli are seen in the nuclear envelope and also in the cytoplasm. These annuli are often found in association with the lamellae; some sections show circular lamellae enclosing groups of annuli. The diameter of these annuli is about 1000 Å, which is within the range reported by Rebhun in his material.

Other basophilic membranes are seen, some arranged in concentric circles, others surrounding granular electron-dense bodies. Within the membrane and around these bodies, vesicles are found.

Cilia have also been found in the adrenal cells of the sea gull.

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Pharmacology of the radula protractor of Busycon canaliculatum. ROBERT B. HILL.

The hearts of many molluscs can be excited by high concentrations of acetylcholine and depressed by lower concentrations. Greenberg has suggested that the demonstration that the former effect is widespread brings mollusc hearts into line pharmacologically with gastropod radula muscle, which is also excited to contract by high concentrations of acetylcholine. However, since isolated radula protractors of *Busycon* do not show spontaneous rhythmicity, previous studies would not have revealed a possible depressing effect of lower concentrations of acetylcholine, which if present, would complete the parallel with cardiac muscle.

Twitches can be elicited from the radula protractor by stimulating the nerve designated 1 by Herrick. A radula protractor *in situ* sometimes possesses spontaneous rhymicity at a frequency of about two per second. Such spontaneous contractions often appeared at the characteristic rate, after or during stimulation producing twitches at a higher or lower rate. Application of 1:4000 nicotine, or cutting nerve 1 between the cerebral ganglion and the point of stimulation abolished spontaneous rhythmicity.

A broad range of concentrations of acetylcholine was tested on radula protractors which were twitching once per second in response to stimulation of nerve 1. The average effect on tonus for each concentration follows, expressed as a percentage of the tetanic contraction resulting from stimulation of nerve 1 at 10 per second: 10^{-2} *M*, 63%; 10^{-3} *M*, 45%; 10^{-4} *M*, 57%; 10^{-5} *M*, 40%; 10^{-6} *M*, 27%; 10^{-7} *M*, 5%; 10^{-8} *M*, 1.5%. The average increase in amplitude of isotonic twitches produced by the lower concentrations was: 10^{-7} *M*, 33%; 10^{-8} *M*, 35.5%; 10^{-9} *M*, 17%. The effects of 10^{-10}, 10^{-11}, and 10^{-12} molar acetylcholine could not be distinguished from those of an equal quantity of sea water.

Thus it appears that the *Busycon canaliculatum* radula protractor lacks the inhibitory half of the biphasic response to acetylcholine found in many molluscan hearts.

Factors in the effects of radiation on the growth rate and conidiation in Neurospora crassa. JOHN KEOSIAN.

Gamma-radiation source: Cs^{137} irradiator, dose rate 5000 r per minute. X-radiation source: 182 Kv machine, inherent filtration of 0.15 mm. Cu, dose rate 4860 r per minute. Incubation temperature: 30° C.

The author stated in a previous abstract that gamma-radiation up to 100,000 r did not produce the characteristic early conidia formation at the irradiated growing frontier of *Neurospora* cultures that could be produced optimally by 9000 r x-radiation. This was attributed at first to two variables. (1) The relative biological effectiveness (R.B.E.) of gamma-radiation *vs.* x-radiation. (2) Falcon plastic *vs.* Pyrex glass. Falcon plastic petri dishes (100 mm.) were used in the earlier work with the Cs^{137} irradiator whose specimen chamber will not accommodate the long, straight tubes used in *Neurospora* growth rate experiments.

The present work revealed the following. (1) A third variable, the age of the culture at the time of irradiation, is a critical factor. Maximum response occurs in 15-hour cultures or older. The response is not appreciable in 8-hour cultures or younger. The cultures used in the earlier work with Cs^{137} were of sub-optimal age. (2) With cultures of optimal age, the results obtained previously in Pyrex glass under x-radiation could be repeated in Falcon plastic dishes under gamma-radiation. (3) The R.B.E. of gamma-radiation for the conidiation effect is about 0.56, while that for the LD 100 is 0.62 or less. (4) The same post-irradiation increase in growth rate reported previously with x-radiation occurs also with gamma-radiation.

Studies on growth rate in the present work with the Cs^{137} irradiator were conducted with specially constructed spiral tubes which would fit into the specimen chamber. Pyrex tubing (13 mm.) was bent into a tight spiral having three coils and an over-all diameter not exceeding 4½ inches. The tubes were numbered and calibrated individually for normal growth rate of unirradiated cultures.

The effect of time of insemination on the development of Fundulus eggs. EVELYN KIVY-ROSENBERG.

During a series of experiments concerned with *Fundulus*, it appeared that embryonic development beyond blastula formation and hatchability depended on the time lapse between insemination and egg stripping. Since the experimental design had involved treatment of uninseminated eggs for various periods of time prior to insemination, the question whether insemination and stripping time were, indeed, intimately related with normal development, arose.

A series of experiments involving samplings of eggs stripped from 25 females between mid-June and mid-July was investigated. Insemination was carried out at chosen intervals between 1 and 145 minutes after stripping. Repeated samples involved time periods between 1 and 20 minutes: relatively few from thirty minutes up. Data indicate that percentage of fertilization and cleavage approaches 100 (*i.e.*, 80–100 with few lower percentages) notwithstanding the time between egg stripping and insemination. The minor differences in fertilizability could probably be traced to the original condition of egg batches. Blastula formation continued in all eggs fertilized. Development to stage 25 (Oppenheimer) was continued only in a small percentage of eggs which had been inseminated 20–30 minutes after stripping. Not all batches of eggs were permitted to develop through hatching, since some were fixed or discarded earlier. However, those which were followed demonstrated that this process was possible even for eggs which had been inseminated up to 15 minutes after stripping, although a much greater percentage were hatched from those eggs which had been stripped 1–2 minutes before insemination.

There appears to be no interference with fertilization and development through blastula formation if insemination is accomplished within two and a half hours after egg stripping but further development to hatching requires that insemination take place within fifteen minutes, but for best results within several minutes of egg stripping.

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Krebs and pentose cycle dehydrogenase systems in the gametes of Asterias as measured with a tetrazolium salt, INT. Evelyn Kivy-Rosenberg, Frances Ray and Natalie Pascoe.

The quantitative, microchemical study of substrate-dependent dehydrogenase system activity was continued: sperm of *Asterias* was compared with eggs (*Biot. Bull.*, **119**: 1960). The same series of 15 substrates was utilized as had been for the egg assays. This includes substrates which require no cofactor as well as those requiring DPN or TPN. Among the substrates were four involved in the Krebs cycle (succinate with no cofactor, malate and alpha-ketoglutarate with DPN as cofactor, isocitrate with TPN as cofactor) and two in the pentose cycle (glucose-6-phosphate and 6-phosphogluconate with TPN as cofactor). The tetrazolium salt which acted as hydrogen acceptor was 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT).

"Dry sperm" was brought up to about a 3.5% suspension by volume and kept iced throughout the pre-incubation period. Incubation was carried out for one hour at 37.5° C. in media containing INT and substrate or appropriate control. Formazan (reduced tetrazolium) was extracted and the quantity measured spectrophotometrically. The substrate-dependent dehydrogenase activity was expressed as micrograms of formazan per milligram of protein.

Of the 15 dehydrogenase systems assayed, malate ranked second in activity; alpha-ketoglutarate possibly third (but not at all consistently); succinate, sixth; isocitrate, ninth; glucose-6-phosphate, seventh; and 6-phosphogluconate among the lowest, *i.e.*, eleventh (possibly due to the type of purity of the particular batch of salt available). For comparison, the rank of activity of egg homogenates for parallel substrates were malate, second; alpha-ketoglutarate possibly first; succinate, thirteenth; isocitrate, third; glucose-6-phosphate, second–third; 6-phosphogluconate, fifth. If rank were considered within the two cycles only, the two obvious differences between activity of sperm and egg dehydrogenase systems are seen in succinate-dependent where in sperm the rank is second, in eggs, sixth; the reverse situation is seen in 6-phosphogluconate (perhaps partially artifact) where sperm ranks fifth and eggs, third.

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The incorporation of nicotinamide-7-C^{14} into pyridine nucleotides of intact eggs and embryos of Spisula solidissima. Stephen M. Krane and Leonard Laster.

The level of diphosphopyridine nucleotide (DPN) in unfertilized eggs of *Spisula solidissima* increases 3–6-fold by incubation in 10^{-4} *M* nicotinamide, whereas levels of DPNH, TPN and TPNH do not change significantly. To determine whether the nicotinamide is incorporated into the DPN, eggs were incubated in nicotinamide-7-C^{14} ($1.4–9.0 \times 10^{-5}$ *M*) in sea water for three hours. DPN and TPN were extracted from the washed cells with trichloroacetic acid, precipitated with acetone, and separated by paper electrophoresis. Labeled pyridine nucleotides were located with a gas-flow scanner, eluted and specific activities were determined. DPNH and TPNH were extracted in hot sodium carbonate solution, oxidized with phenazine methosulfate, isolated and assayed as above. More than 90% of the pyridine nucleotide radioactivity was in DPN. Ratios of C^{14} content of DPN:TPN were 84 and 78 in two experiments, whereas those for DPNH:TPNH were 1.9 and 1.7. Specific activities of DPN:DPNH:TPN :TPNH were in the ratios 1.11:1.00:0.20:0.32 and 1.03:0.98:0.47:0.38. Carbon^{14} which migrated on electrophoresis with authentic nicotinamide was recovered in the acetone supernatant after precipitation of the nucleotides. Its concentration was greater than that in the incubation medium, suggesting active transport of nicotinamide. Incorporation of nicotinamide-7-C^{14} into pyridine nucleotides of three-hour fertilized eggs and 24-hour embryos was also observed, but specific activities were not determined. These studies demonstrate that intact *Spisula* eggs and embryos take up nicotinamide and incorporate it into pyridine nucleotides. The lower specific activities of TPN and TPNH are consistent with their derivation from DPN. In addition, the nearly equal specific activities of DPN and DPNH on one hand, and TPN and TPNH on the other, suggest that only a relatively small fraction of the DPN is converted to TPN during the time period studied.

Triphosphopyridine nucleotide formation and disappearance in the presence of extracts of eggs, embryos and adult liver of Spisula solidissima. LEONARD LASTER AND ROBERT K. CRANE.

To explore whether an increase in DPN kinase activity is associated with the observed alteration in TPNH concentration of *Spisula solidissima* eggs after fertilization, this enzyme has been studied. Activity was assayed by determining TPN formation using a TPN-specific preparation of glucose-6-phosphate dehydrogenase. Kinase activity of egg homogenates was linear for one hour and was proportional to enzyme concentration in the range used. Requirements for DPN, ATP and Mg^{++} were demonstrated. Most of the kinase of homogenates centrifuged with particles recovered at 600 *g*. Solubilization was achieved by freezing and thawing. Highly approximate K_m values were determined: ATP, 8.5×10^{-3} *M* and DPN, 1.5×10^{-4} *M*. Kinase activity per unit volume of 18-hour embryos and per unit weight (wet) of adult liver was not greater but somewhat less than kinase activity per unit volume of unfertilized eggs. The soluble fractions of embryo and liver homogenates contained a greater percentage of the whole homogenate's kinase activity than did that of eggs. The assay for DPN kinase was complicated by the presence of an enzymatic activity in eggs and embryos that caused the disappearance of TPN added to homogenates. This activity was stimulated by addition of DPN and Mg^{++}. It remained predominantly in the soluble supernatant of centrifuged homogenates. In contrast, liver-soluble supernatant caused added TPN to disappear quite rapidly without added cofactors and this disappearance of TPN was suppressed by the addition of DPN.

Metabolic pathways in the dogfish and skate lens. SIDNEY LERMAN, JEANNE FONTAINE AND KENNETH WOODSIDE.

A comprehensive study of carbohydrate, protein and RNA metabolism was performed on lenses derived from dogfish of various ages ranging from the foetal dogfish lens (approximately 25 mg.) to the mature lens (approximately 1500 mg.). The results of these investigations indicate that there is a very marked increase in albuminoid RNA of the dogfish lens as it ages, while microsomal and soluble RNA remain relatively unchanged. The turnover of these RNA fractions shows a similar pattern and there is a close correlation between these results and the relative rates of amino acid incorporation into the soluble and insoluble lens protein fractions. However, there is little if any measurable RNAse activity in any of these lenses.

While protein and RNA metabolism in the dogfish lens show an aging pattern that is similar to certain mammalian lenses (rat and rabbit), there is quite a marked difference in carbohydrate metabolism. In the latter (rat lens) there is an active hexose monophosphate pathway of glucose metabolism in the young and rapidly developing lens, which diminishes in activity and importance as the lens ages. Studies on the dogfish lens indicate that glycolysis is the major pathway of glucose oxidation in lenses derived from dogfish of any age group, while glucose oxidation via the direct oxidative pathway occurs to a negligible extent.

Preliminary investigations on the skate lens indicate that carbohydrate as well as RNA and protein metabolism are more closely akin to the mammalian lens than the dogfish lens.

A comparative study of dopa oxidase systems in marine invertebrates. Herman W. Lewis.

Analysis of the dopa oxidase system of *Drosophila* has revealed a complex system, one component of which is an activating enzyme. Similar complexity has not been found in lower forms, offering the possibility that this enzyme system may be a useful tool for investigating evolution at the biochemical level. To gain insight into such evolution, dopa oxidase systems are being surveyed among the invertebrates. This report describes preliminary findings in representative samples of a few phyla, with respect to the presence or absence of an extractable activating enzyme of dopa oxidase. Activated dopa oxidase is stable at room temperature but *in vitro* activation occurs only in the cold, presumably because the activating enzyme is attacked by proteolytic enzymes at room temperature. During incubation in $M/15$ phosphate buffer, pH 7.0 at 0° C., aliquots of the crude extract are periodically removed and added to a solution of 0.67 M dopa. The presence of active dopa oxidase results in the production of dopachrome, which is measured spectrophotometrically. The increase in optical density at 475 mμ per minute is used as the measure of dopa oxidase activity. The presence of an activating enzyme is indicated when an extract has no or little activity shortly after extraction but shows an increase in activity, following a sigmoid-shaped curve, when activity is plotted against time. This situation is found in *Henricia sanguinolenta, Callinectes sapidus, Carcinides maenas, Cancer irroratus, Pagurus pollicaris, Libinia emarginata, Limulus polyphemus, Palaemonetes vulgaris, Loligo pealeii, Mercenaria mercenaria, Aequipecten irradians, Busycon canaliculatum, Phascolosoma gouldii, Metridium* sp. and *Microciona prolifera.* In *Asterias forbesi* and *Crassostrea virginica* the dopa oxidase was fully activated when extracted. In the concentrations used and the tissues extracted, no dopa oxidase has been found in the following: *Ophioderma brevispina, Arbacia punctulata, Strongylocentrotus dröbachiensis, Echinarachnius parma, Chaetopleura apiculata, Crepidula fornicata, Polinices duplicata, Polinices heros,* and *Thais lapillus.*

Separation of an insulin-containing fraction from the islet of the goosefish. Arnold W. Lindall, Jr. and Arnold Lazarow.

Previous investigations have suggested that most of the insulin in a fish islet homogenate is removed by centrifugation; it is recovered in the mitochondrial fraction.

Islets (50–100 mg.) were homogenized in 0.25 M sucrose and separated into nuclear (I), mitochondrial (II), microsomal (III), and supernatant (IV) fractions. Fraction II contained 80% of the cytochrome oxidase activity and more than 75% of the total insulin, as determined by paper chromatography of the purified acid alcohol-soluble protein (ASF), immunoassay and blood sugar-lowering.

Fraction II was further subfractionated by centrifugation (2 hours at 100,000 g and 0° C.) using a continuous linear density gradient (1.0–2.0 M sucrose). The gradient was separated (from the bottom) into 18–20 subfractions of 10 drops each. The protein was distributed into a bimodal curve with peaks at densities of 1.205 and 1.173 gm./cc. of sucrose. The high density component (1.205) contained about 80% of the total protein present in fraction II. The cytochrome oxidase activity coincided with the low density protein component (1.173), and was completely separated from the high density protein component. The distribution of the purified acid alcohol-soluble protein coincided with the high density component; more than 75% of this purified ASF migrated with the same R_f as bovine insulin. Furthermore, chromatography showed that while large quantities of "insulin" were present in the high density component (1.205), none could be detected in the component containing the cytochrome oxidase activity (1.173). These findings suggest that the insulin-containing (secretion) granule can be separated from the cytochrome oxidase granule (mitochondria).

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Intracellular cardiac potentials in Limulus during ganglionic stimulation. FRANCES V. MCCANN AND DONALD W. MILLER, JR.

The cardiac action potential of *Limulus* is distinctive because of the barrage of small potentials which persist during the prolonged phase of repolarization or "plateau." The neurogenic nature of the origin of the heart beat in *Limulus* suggests that the ganglion continues to discharge during the plateau, but its contribution to the maintenance of this phase of the cardiac action potential remains obscure. Recordings of electrical activity in single cardiac cells with conventional microelectrodes during spontaneous and driven activity indicate that as the frequency of stimulation increases, the plateau shortens markedly with a consequent shortening of the diastolic phase. As the frequency is further increased, the plateau is completely obliterated, and the ganglionic discharge is no longer evident. At stimulation frequencies greater than three times the normal heart rate, a new level of polarization is established (12 mV), which is much less than the original level (48 mV), and the action potential duration and magnitude are markedly diminished.

The nature of the pigments in the integument and eye of the hermit crab, Pagurus pollicaris. JOHN J. MCNAMARA, GEORGE SZABÓ AND R. T. SIMS.

This investigation was made as part of a project to find ommochromes in the Crustacea. The hermit crab is a useful animal because of its soft integument. The tissues were ground in filtered sea water and then extracted consecutively with acetone and acid methanol to remove carotenoids and ommochromes, respectively. The integument contained a large amount of carotenoids but no ommochrome. The eyes contained a small amount of carotenoid and a large amount of red ommochrome. This ommochrome showed the typical redox behavior characteristic of this pigment and became straw-colored on the addition of sodium thiosulfate.

Histological sections, prepared from paraffin-embedded integument, showed no ommochrome. Also, no pigment granules were revealed by the hexamine silver and Schmorl's techniques. The orange carotenoid granules were shown to be in dendritic chromatophores by mounting pieces of integument whole in glycerine jelly. They were stained by histochemical tests for lipid. Granules of a blue pigment were seen in the unstained preparations. The blue color disappeared when the tissue was boiled, so it is suggested that this pigment is a carotenoprotein.

Both eyestalks were removed from the animals and the chromatophores observed over a period of seven days. No effect was demonstrable.

It is concluded that the eye of *Pagurus pollicaris* contains ommochrome and carotenoid pigments and the integument contains only carotenoids.

Action potentials in single cells of a tunicate heart. DONALD W. MILLER, JR. AND FRANCES V. MCCANN.

The tunicate heart is known to exhibit the phenomenon of beat-reversal, *i.e.,* the origin of the heart beat may occur at either end of the V-shaped heart and thus pump blood alternately forward or backward. The site of origin of the beat is generally believed to be localized at either pole of the heart, and thus pacemaker activity is described as "bipolar." Intracellular recordings of spontaneous electrical activity in single cells of *Ciona intestinalis* myocardium were studied with conventional microelectrodes less than 0.5 μ outside tip diameter. A portion of the tunic was removed, and a very small incision was made through the pericardium to expose only the area of electrode impalement. The maximum resting potential recorded was 48 mV, and action potentials reached a maximum value of 50 mV. There was no significant overshoot or delayed period of repolarization (plateau) in the polar or interpolar regions of the heart. At a heart rate of 60 beats/minute, pacemaker depolarization persists for 500 msec., and threshold for the rapid upstroke of the action potential occurs at 5 mV, or approximately 25% depolarization. That the origin of the cardiac action potential is myogenic is supported by this observation.

Experiments on interspecific fertilization between Ciona, Styela and Molgula (ascidians). A. MINGANTI.

Eggs and sperms of the ascidians *Ciona intestinalis* (Linnaeus), *Styela partita* (Stimpson) and *Molgula manhattensis* (De Kay) have been crossed with each other in the six possible combinations. Mature eggs were obtained from the oviducts or the gonads. They were deprived of their membranes with steel needles, and inseminated with a dense sperm suspension of another species. From 1% to 10% of the eggs, according to the crosses, were activated by the foreign sperm. No activation ever occurred in eggs still enveloped by their membranes. Two eggs out of 700 in the cross *Ciona* ♀ × *Styela* ♂, and one egg out of 350 in the cross *Ciona* ♀ × *Molgula* ♂, cleaved normally until a pregastrular stage; they did not gastrulate or differentiate, and cytolyzed after some hours. In all other cases the activation brought about continuous changes, going on for many hours, in the egg shape and in the distribution of the pigmented granules. No polar bodies were formed, although many eggs produced hyaline lobes that were soon reabsorbed. In the activated *Ciona* eggs which did not develop as described above, abortive cleavages were also observed. In such eggs a cytological study revealed an abnormally high number of chromosomes, possibly due to endomitosis. In the activated *Molgula* eggs many sperm heads of either *Ciona* or *Styela*, not resolved into chromosomes, were seen.

Malic dehydrogenases of developing Arbacia embryos. RICHARD O. MOORE AND CLAUDE A. VILLEE.

Arbacia embryos grown in sea water at 20.5° were harvested by centrifugation after 6, 12, 24, or 48 hours. Any group in which less than 95% of the eggs were fertilized was discarded. The embryos were homogenized in 0.025 *M* barbital buffer, pH 8.7, and centrifuged at 10,000 *g* for 20 minutes. The supernatant fraction was mixed with starch granules to a thick paste, inserted 5 cm. from the cathode in a 1 × 15 cm. slit in a 33 cm. long starch gel block and subjected to electrophoresis (10 hours, 200 v, 45 mA, 0.025 *M* barbital, pH 8.7). After electrophoresis, 1-cm. sections were cut, eluted with artificial sea water and centrifuged. The supernatant fractions were assayed for malic dehydrogenase activity, using DPN, 3-acetyl pyridine DPN and thionicotinamide DPN.

Unfertilized eggs have five DPN-malic dehydrogenases, numbered I to V in order of migration toward the anode. Peak II, the major one representing 60% of the total activity, migrates about 6 cm. from the origin. In 6-hour embryos only peaks I, II and IV could be detected. Twelve- and 24-hour embryos have peaks I, II, IV and V; 48-hour embryos have the same four, but peak V is relatively larger than at 12 or 24 hours. The ratio of malic dehydrogenase activity with APDPN and DPN in embryo extracts not subjected to electrophoresis changes from 0.68 in the unfertilized egg to 2.2 in the 48-hour embryo. The TNDPN/DPN ratio decreased from 0.86 in the unfertilized egg to 0.22 at 6 hours and then increased to 0.55 at 48 hours.

The malic dehydrogenase activity with APDPN migrated differently from the DPN enzymes and some fractions had only DPN, others had only APDPN, activity. As many as 7 peaks with APDPN activity were observed. The ratio of APDPN:DPN activity differs among the peaks. Ratios of activity with DPN analogues in simple tissue extracts may be misleading, for they may represent the sum of several individual enzymes with varying analogue ratios. In the course of these investigations an enzyme with D-malic dehydrogenase activity with APDPN, but not DPN, was discovered. This migrates electrophoretically at a different rate from the L-malic dehydrogenases.

Studies on the isolated islet tissue of the toadfish (Opsanus tau): aldolase content of islet and other tissues. JOSEPH F. MORAN, JR.

As a part of a systematic study of the metabolism of the isolated islet tissue of the toadfish and because of the known effect of glucose on the release of insulin from the beta cell we have been investigating the enzymes involved in the metabolism of glucose by islet tissue. Previously we have measured the glucose-6-phosphate dehydrogenase (G-6-PD) and 6-phosphogluconate dehydrogenase (6-PGD) contents of toadfish tissues. In the present study the aldolase content of toadfish tissues was determined by measuring the rate of reduction of diphosphopyridine nucleotide (DPN) at 340 $m\mu$ in a Beckman spectrophotometer under standardized conditions. Weighed samples of islet (1–5 mg.) and other tissues were homogenized in glycylglycine buffer and aliquots (10–20 μl.) were added to the assay system; the final volume was 300 μl.

The average aldolase content of the islet tissue of 10 animals was 57 μM DPN reduced per gram wet weight of tissue added per hour; this was the lowest of any tissue examined. The aldolase contents of liver, heart, gill and testis were about 1.5 times greater, that of kidney was twice as great, whereas brain and muscle had 5 times as much aldolase as did islet tissue.

Although islet has about equal amounts of aldolase, G-6-PD, and 6-PGD, other tissues differ in their relative enzyme contents. For example, muscle has little, if any, G-6-PD or 6-PGD activity, but it has the highest aldolase activity. Similarly, the aldolase content of brain is 2 to 4 times greater than that of G-6-PD and 6-PGD, whereas the aldolase content of all other tissues examined was only 25–50% of the G-6-PD content.

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Long refractory periods of branchial sensory nerve endings in dogfish. RICHARD W. MURRAY.

Nerve impulses originating at sensory endings in the skin of the pharyngeal face of the gill bars of *Mustelus canis* were recorded in fine strands of a pre-trematic (*i.e.*, pure sensory) branch of the vagus. Electrical stimuli (E) were applied through wick electrodes, one over the ending and the other indifferent; the stimulating current was monitored. Mechanical stimuli (M) were given by a probe attached to a loudspeaker.

The time-course of relative refractoriness was followed, using paired stimuli, either E-E, E-M or M-E. Paired mechanical stimuli were not used because of possible changes in the effectiveness of the second stimulus due to the deformation of the skin caused by the first. Refractoriness following an antidromic impulse (A) was also tested (A-E and A-M).

The E-E combination gave the following time-course for relative refractoriness (the strength of the second stimulus is expressed as a percentage of the threshold for single shocks, with its S.D.; 22 units; temperature 21°–24° C.): 20 msec., 173 ± 13; 30 msec., 162 ± 12; 40 msec., 155 ± 11; 60 msec., 145 ± 10; 80 msec., 139 ± 9; 100 msec., 134 ± 9; 150 msec. 119 ± 5; 200 msec., 113 ± 6; 250 msec., 106 ± 3. All the other combinations gave comparable values.

The long time-course found here, unlike that in axons, supports the argument that refractoriness in the classical sense (as tested by paired stimuli) can be one of the factors controlling the frequency of the repetitive discharge of a sense organ, even at the low rates of firing which are commonly found.

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Nitrogen inhibition of active absorption of D-glucose in fish intestine. X. J. MUSACCHIA AND D. D. WESTHOFF.

In vitro preparations of everted intestinal sacs from the marine fish, *Stenotomus versicolor,* and the fresh-water fish, *Ameiurus nebulosus,* were used to measure active absorption of D-glucose. Seventy *A. nebulosus* and 75 *S. versicolor* preparations were run in the experiments. In order to induce inhibition by anaerobic conditions, nitrogen gas (100%), was flushed continuously through the incubation medium, either fresh-water or marine teleost Ringer's. In the intestinal segments (whole intestine, or upper and lower areas) of *A. nebulosus* with both 5 mg.% and 10 mg.% D-glucose, active absorption continued during the 30-minute incubation period. Small amounts of endogenously produced glucose with and without nitrogen were confirmed with blank tests. Blank tests consisted of complete experiments without D-glucose added to the Ringer's incubation medium. These tests were necessary to substantiate that under anaerobic conditions, some active absorption occurred.

By way of comparison, intestinal segments from the marine fish, *S. versicolor,* under nitrogen anaerobiosis differed considerably. In both upper and lower segments there was uptake of 10 mg.% D-glucose by both the mucosal and the serosal surfaces. It was concluded, therefore, that nitrogen was an effective inhibitor of active absorption of glucose.

Comparisons of active absorption in upper ("duodenal") and lower ("ileal") segments with 5 mg.% and 10 mg.% D-glucose were made. The upper intestinal segments from the catfish, *A. nebulosus,* showed the greater levels of active absorption with 10 mg.% D-glucose in the medium, in terms of μM glucose/gm. dry wt./30 min. Average values were mucosal uptake, 21.36 and serosal transfer, 8.45. The upper and lower intestinal segments in the scup, *S. versicolor,* were comparable in active absorption of 5 mg.% and 10 mg.% D-glucose.

Phlorizin inhibition of active absorption of D-glucose in fish intestine. X. J. MUSACCHIA AND D. D. WESTHOFF.

Inhibition of active absorption with phlorizin at the cell surface level of the "lumenal" intestinal epithelium has been well documented in mammalian preparations.

Phlorizin, 5×10^{-4} *M,* placed on both sides of intestinal wall in catfish, *A. nebulosus,* preparations resulted in inhibition of active absorption. In fact, endogenously produced glucose was added to the medium on both sides, from 11.38 to 13.32 μM/gm. dry wt./30 min. on the serosal side, and from 17.09 to 27.53 μM/gm. dry wt./30 min. on the mucosal side. The levels of inhibition were comparable with each of 16 upper segments with 10 mg.% D-glucose. When phlorizin was only added to the mucosal side, there continued to be endogenously produced glucose added to the serosal medium but there was no uptake from the mucosal medium. Thus, phlorizin inhibits active absorption in intestine of catfish.

In the intestinal preparations from scup, *S. versicolor,* the action of phlorizin, 5×10^{-4} *M,* differed somewhat. For example, when phlorizin was placed on both sides of the intestinal wall there was uptake from the mucosal and the serosal side as well. Thus, inhibition of active absorption was primarily in terms of glucose transferred. These differences in transport mechanisms in the marine and fresh-water fish intestinal preparations suggest the presence of at least a two-step process in active absorption in fish intestine.

There were 40 experiments with catfish preparations and 36 experiments with scup. Each experiment consisted of the upper segment under phlorizin inhibition and the lower segment as controls, without phlorizin in the incubation medium.

A persistent diurnal phototactic rhythm in the fiddler crab, Uca pugnax. JOHN D. PALMER.

In simple tests designed to establish the sign of the phototactic response of fiddler crabs it was found that the crabs sometimes responded positively and sometimes negatively. These opposing results suggested the possibility of a rhythmic sign reversal in responsiveness to light.

To test this, small, rectangular, aluminum pans were constructed and the tops covered with transparent plastic covers. One half of each cover was painted flat black as was the inside of the pan beneath this area. These pans, each containing a single crab, were centered on a fulcrum so that they would tip slightly in one direction when the crab moved into the lighted portion and the other direction when the crab moved into the darkened end. A thread connected each pan to a kymograph pen, and the position of the crabs within the pans was recorded continuously during July and August, 1962. Over 11,000 individual, one-hour observations were made in this manner. The experiment was conducted in a constant temperature room (18° C.) at a constant light intensity of 250 foot candles. Fresh crabs were substituted every 15 days.

A persistent daily rhythm was found in the length of time spent in the light and dark ends of the pans (the "preference" being a measure of phototactic response). Between 5 AM and 8 AM the crabs spent up to 70% of each hour in the lighted end of their pans. This response gradually decreased to 50% for the hours between 3 PM and 7 PM. Between 7 PM and the early morning hours the crabs spent the major part of each hour in the darkened end of the pan.

Extrapolating to the natural habitat, at sunrise and during the cool early morning hours, fiddler crabs are quite strongly attracted to light and they emerge from their burrows. During the remaining hotter part of the day, when desiccation problems increase, the attraction to light becomes less intense. At sunset the crabs become photonegative and return to their burrows.

The persistence of a biological rhythm in continuous bright illumination. JOHN D. PALMER, CHARLES S. YENTSCH AND SUSAN A. DEROPP.

It is well known that most plants do not tolerate uninterrupted strong illumination. There are, however, a few algae such as *Chlorella, Scenedesmus,* etc., which grow well under these conditions, and these, therefore, have been extensively studied. It is also well documented that continuous bright light inhibits the expression of biological rhythms in both plants and animals. The question was raised as to whether algae which were known to grow well in continuous light might also maintain rhythmic variations in their growth processes.

The green alga *Nannochloris* has been found to grow quite well in continuous illumination and was used in the following experiment. A turbidostat was placed in a constant temperature cabinet (21 ± 1° C.) and provided with unilateral illumination from a bank of fluorescent lights (700 foot candles). To monitor the amount of light passing through the culture, a photocell was attached to the side of the growth chamber away from the light source. When the cell number surpassed a prescribed density, nutrient automatically flowed into the growth chamber, flushing out a sufficient number of cells to return the optical density to the prescribed level. The amount of nutrient added was thus a measure of the rate of cell division per unit time. Each time new nutrient was added the time of addition was automatically recorded and this record was used to indicate the rates and times of cell divisions. Data were gathered between 13 July and 31 July, 1962.

A persistent solar-day rhythm was found in the rate of cell division. The division rate remained relatively constant through the late afternoon, night time and early morning, rose sharply to a maximum at noon and gradually returned to the preceding constant level by 6 PM. More than a two-fold increase in the rate of cell division occurred at noon and over half of the daily cell divisions took place between 9 AM and 5 PM.

Gel-sol transformations in the unfertilized egg of Arbacia punctulata. ARTHUR K. PARPART AND THOMAS V. N. BALLANTINE.

Gel-sol transformations of the cytoplasm of the unfertilized egg of *Arbacia punctulata* have been studied by numerous investigators under a great variety of conditions. The present report comprises studies on the independent motion of the echinochrome granules and of the fine cytoplasmic streaming observed by television microscopy. Cysteine, in concentrations of 0.005 *M* to 0.0005 *M* dissolved in sea water, was observed to markedly decrease both these motions in 30 to 180 seconds. By 7 to 15 minutes all cytoplasmic motions, except Brownian, of minute particles was stopped. By the sucrose flotation method, centrifugation at 18,000 *g* for 30 seconds of eggs thus exposed to 0.005 *M* cysteine in sea water presented a picture of complete immovability of the egg particulates, while untreated eggs gave the normal sedimentation pattern and a number of quarter and half eggs. The gelation of the egg cytoplasm induced by 0.005 *M* cysteine in sea water invariably leads to activation of the egg, and the ensuing gel-sol changes follow those of an activated egg, if the egg is re-exposed to sea water within 10 to 15 minutes. Longer exposures produce irreversible gelation. Other agents, *e.g.,* 1 *M* sucrose and EDTA in sea water, also produce a gelation of the cytoplasm but this is readily reversible on re-exposure to sea water, and never produces activation.

It is doubtful that cysteine or sucrose or EDTA penetrate the egg cytoplasm in the short time required for gelation in these compounds. It is suggested that changes in the plasma membrane of these eggs induced by these compounds lead to a wave of gelation throughout the cytoplasm so that particles which are normally moved about the egg by fibers (echinochrome granules) and cytoplasmic streaming are prevented.

Electron microscopic observations of secretory granules in the adhesive surface of Hydra pirardi. DELBERT E. PHILPOTT AND ALFRED B. CHAET.

Previous work on the starfish tube foot has revealed a correlation between the ultrastructure of the fibrous-ellipsoidal "secretory packets" and the adhesive properties of this organism. The present report deals with a similar investigation which attempts to observe the ultrastructure of the basal portion of *Hydra pirardi,* and to correlate, if possible, these findings with its adhesive ability.

Electron micrographs reveal small spherical structures, 1–1.5 microns in diameter, which are located in the epidermis of the distal portion of the peduncle and are more abundantly found in the basal disc itself. Many of these structures (secretory granules) appear as loose "balls of yarn" surrounded by one or two outer shells. Others are more homogeneous in appearance and are also surrounded by an outer shell. As the matrix of these secretory granules becomes more homogeneous in nature, the vacuolar space around them, which is bounded by a double membrane, gradually disappears.

Mitochondria are found interspersed between the granules, and Golgi bodies frequently are found in association with them. Although the mode of release of these granules has yet to be conclusively determined, preliminary evidence suggests that the individual structures bulge against, and are finally extruded through, the cell membrane into the external environment.

Since light microscopy studies have demonstrated that these secretory granules are PAS- and alcian blue-positive, and since these granules are found mainly on the attaching surface, it is hypothesized that they are used in the adhesion of *Hydra* to a substratum. There is an interesting similarity in the staining properties and in the ultrastructure between the secretory granules of *Hydra* and the "secretory packets" previously demonstrated in the starfish tube feet.

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Chromatophores of decapod Crustacea in hypodermal organ culture. NANCY PIANFETTI, JUDITH HICKMAN AND RICHARD C. SANBORN.

Organ cultures of the hypodermis of *Homarus americanus, Cancer irroratus, Crangon septemspinosus, Callinectes sapidus, Libinia emarginata,* and *L. dubia* have been prepared. The media used contained balanced crustacean saline solution, organic acids, carbohydrates, lactalbumin hydrolysate, 10 to 20% *Callinectes* or fetal calf serum, and antibiotics. In certain of these media, the hypodermis survives without visible morphological change or growth for at least 60 days when the medium is changed at seven- to ten-day intervals.

Chromatophores of such cultured hypodermis behave differently from those of animals from which the eye-stalks have been removed. For example, following eye-stalk ablation the melanophores of *Callinectes* contract to stage 1 while the erythrophores expand to stage 5. In culture, both types remain contracted (stages 1 or 2). *In vivo,* following eye-stalk removal, the melanophores of *Crangon* uropods and telson are first expanded (stage 4 or 5) then contract while the body chromatophores contract and then expand to stage 4 or 5. *In vitro,* both groups of chromatophores remain in stage 2.

An exception to the contraction of *Crangon* chromatophores *in vitro* is noteworthy. The melanophores of the hypodermis of whole eye-stalk explants are dispersed rather than contracted. This suggests that under our culture conditions the chromatophores are susceptible to humoral factors. Attempts to test this hypothesis using other hypodermis explants have not, however, shown changes under the influence of extracted, boiled, or alcohol-soluble or -insoluble preparations of eye-stalks. These same preparations act in the usual fashion on the chromatophores of *Uca in vivo,* both before and after incubation with hypodermal explants.

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The chloride permeability of crayfish muscle fibers. JOHN P. REUBEN, LUCIEN GIRARDIER AND HARRY GRUNDFEST.

Electrical and volumetric data on single fibers indicate that the membrane of crayfish muscle is permeable to Cl, and the sites for Cl movement appear to be distinct from those permeable for K. Under normal conditions the changes in membrane potential caused by altering the level of external Cl are much faster than those produced by changing K. Raising the pH to 10 does not affect the membrane potential, but the theoretically expected response (58 mv./decade) is obtained on changing the external K, while changing the Cl affects the potential little or none. Thus, it appears that Cl penetrates through positively charged membrane sites whose specificity for Cl decreases with decrease of the titrable positive charge density.

Crayfish fibers normally exhibit marked hyperpolarizing rectification due to increased Cl conductance. This rectification is abolished by depleting intracellular Cl, by high pH, or by applying picrotoxin, the effective resistance to hyperpolarizing currents rising accordingly. At pH 10 the length constant also rose from 3.15 to 4.22 mm. GABA, which normally increases conductance, did not affect the current-voltage curves of fibers with depleted Cl, and there was no change on applying picrotoxin. Hyperpolarizing rectification is also abolished by removing the Cl with inward current delivered through a microelectrode filled with 3 M K propionate as the intracellular cathode. Hyperpolarizing responses, which under normal conditions never occur in crayfish muscle fibers, are elicited regularly when the Cl is depleted, and applications of GABA do not block the response. Since the latter result from hyperpolarizing K inactivation, the K- and Cl-selective channels must have distinctive pharmacological properties.

GABA and picrotoxin affect the rates of water movements that are produced by altering the ionic environment of the fibers. The swelling of fibers which are transferred from the standard medium to one containing 153 meq./l. K (isotonic) was 5-fold slower in picrotoxin than in GABA. Since the electrophysiological data indicate that picrotoxin blocks Cl permeability, this result strongly suggests that local currents are set up by the movement of ions through specific sites permeable to K and Cl, respectively.

Some properties of stellarin, the photosensitive pigment of the starfish, Asterias forbesi. MORRIS ROCKSTEIN.

Careful control of the temperature of extraction, the pH of the 2% digitonin extracting solution and the temperature during exposure to light conclusively proved the presence of a photosensitive pigment in the dorsal skin of the starfish, *A. forbesi.* When extracted at low pH Teorell buffer solution of digitonin, the pigment assumed a true violet hue with an absorption maximum at 567 mμ, but failed to exhibit any photolability. Alkalinization of this extract with 1 N NaOH (from a pH of 4.8 to 12.4) converted the pigment to a now-photosensitive, orange-peach pigment with an absorption maximum at 485 to 490 mμ. This was similar to the extract which could be made of the pigment from aqueous suspension by an alkaline Teorell buffer solution of digitonin (pH 12.0) as the primary extracting medium. Intermediate pH values for the digitonin solutions employed in extracting this pigment yielded intermediate colors, varying from violet-peach to peachy-violet, and possessing intermediate absorption maxima as well. Pronounced and highly reproducible difference spectra were obtained for all alkaline digitonin pigment extracts, exposed for short periods of time to white light of medium to moderate intensity under controlled temperature and buffered pH values of the extracting medium. This difference spectrum possesses a maximum at 576 and a minimum at about 485 mμ. One can summarize these data by stating that the photosensitive pigment of this species, stellarin, is a pH indicator-like substance, which, in the alkaline range, is unstable in light and in the acid range is stable to light effects. This susceptibility to light in the alkaline range is readily eliminated by acidification and the stable form of the pigment at low pH is readily converted to the photolabile form upon alkalinization.

Ultraviolet damage to the cortex of the sea urchin egg. RONALD C. RUSTAD.

Various types of radiation interfere with the fertilization reaction. The elevation of the fertilization membrane and the differentiation of the hyaline layer are known to be suppressed on the hemisphere of a sea urchin egg which faces an ultraviolet lamp.

Phase contrast observations on U.V.-irradiated cells indicate that the round cortical granules can be transformed into an irregular splotchy shape. This change often does not occur until after fertilization, when normal granules yield fine filaments.

The irradiated eggs elevate a highly birefringent fertilization membrane on the cytoplasmically-shaded side. At high doses there is no detectable change in the weak birefringence at the irradiated surface following insemination. Hence, not even a "tight fertilization membrane" is formed on the damaged side.

Pigment granules sometimes migrate preferentially to the cortex of the directly-irradiated hemisphere of the unfertilized egg.

These observations indicate that U.V. damages some component of the cortex of the sea urchin egg and prevents the normal breakdown of the cortical granules. The granule material is not extruded into the perivitelline space to aid in the elevation of the fertilization membrane or in the formation of the final fertilization membrane structure.

Drugs causing localized lightening and darkening of the common sand dab, Scophthalamus aquosus. George T. Scott, Richard L. Clark and James C. Hickman.

Preliminary dose-response observations were made by injecting 0.05 ml. of the dilutions of the drugs subcutaneously in the flank of one or two fish. Finally, three injections adjusted about the approximate effective dose (ED) were injected into five fish. The amount of drug required to produce a distinct light or dark patch at least one half inch in diameter in four out of five fish was taken as the ED_{80}.

The ED_{80} in μg. for drugs producing localized chromatophore aggregation were as follows: epinephrine 1, norepinephrine 1, isopropylarterenol 10, serotonin 100, phenelzine 400, pheniprazine 400.

Drugs producing localized darkening fell into several classes. The ED_{80} in μg. were as follows: (local anesthetic) dibucaine 2; (tranquilizing) eleven phenothiazine ataraxics ranged from 10 to 150 (the most active was mepazine and the least active was chlorpromazine sulfoxide) reserpine phosphate 30, isoreserpine phosphate 60, meprobamate 500; (sedatives) meperidine (Demerol) 40, ethchlorvynol (Placidyl) 200; (serotonin-blocking) lysergic acid diethylamide 500; bimaleate 500; (adrenaline-blocking) phenoxybenzamine 10, N-(2-chloroethyl) dibenzylamine 10, dichloroisopropyl arterenol 100; (antidepressant) imipramine 12. Barbiturates were found to be inactive.

The high potency of the epinephrines in producing localized lightening, together with the observation that the epinephrine blocking agents are active chromatophore dispersers, suggests that a physiologically active amine secreted by the aggregating nerve fibers is the chemical mediator. Pheniprazine and phenelzine may act by monoamine oxidase inhibition.

No evidence of dispersing nerve fibers or pituitary control of chromatophores has been found. Therefore, the drugs causing darkening may be acting by an adrenolytic property at the chromatoneural junction or acting directly on the melanocyte cell.

This study was aided by a National Institute of Mental Health grant MY-3903 to Oberlin College.

Resistance to gamma irradiation of fertilized eggs of Arbacia correlated with their stage of development. Carl Caskey Speidel and Ralph Holt Cheney.

Fertilized eggs of *Arbacia* at 13 time-stages of development were subjected to equal gamma irradiation at 5-minute intervals, as follows: time in minutes after insemination; 2, 7, 12, 17, 22, 27, 32, 37, 42, 52, 57, 62. In Experiment A, the dose at each time-stage was 4 kr; in B, 8 kr; C, 12 kr; D and E, 16 kr; F, 24 kr; G, 32 kr. Four experiments included three later time-stages of 72, 82, and 92 minutes.

Comparative irradiation damage was best estimated from cultures of thousands of embryos observed at (1) 7–13 hours, for differences in hatching time and motility, (2) 24–48 hours and longer, for motility, differentiation, injury and viability. Delays in first and later cleavages were also noted. The most resistant time-stages were those from which embryos developed that displayed earliest and greatest amount of motility, least degree of injury, and best differentiation and viability. Based on these criteria, 12 time-stages from fertilization to first cleavage were arranged in order from most radio-resistant to most vulnerable, as follows: 32, 27, 37, 42, 47, 22, 52, 57, 2, 7, 12, 17.

Eggs in the most resistant time-stages (32, 27, 37) were in the streak phase, approximately midway between early vulnerable time-stages (17, 12), characterized by the monaster, and later vulnerable time-stages (57, 52), characterized by the end phases of mitosis and the onset of first cleavage. Eggs in time-stages 7 and 2, also very vulnerable, contained separate pronuclei. Time-stages 22, 42, and 47 were transitional.

Comparison of unequal radiation dosages showed that a one-unit dose to eggs in vulnerable stages of monaster (17, 12), separate pronuclei (7, 2), and first cleavage onset (57), induced more damage in the developing embryos than a two-unit dose to eggs in resistant streak stages (27, 32).

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Amino acids in the economy of the bamboo worm, Clymenella torquata. GROVER C. STEPHENS.

An analysis of the amino acids in sea water was made using a Spinco Model 120 amino acid analyzer. Sea water was obtained at low tide from a mud flat where *Clymenella* was abundant. An attempt was made to obtain sub-surface water by digging a few inches below the surface at the water's edge. Samples were frozen at the time of collection and were later evaporated to dryness and the resulting salt extracted with 2% HCl in acetone. Eleven neutral and acidic amino acids were identified. Total amino acid concentration was 74 micromoles per liter. Major components were glutamic acid (25.1 micromoles), alanine (15.8), glycine (9.64), and aspartic acid (8.65). These figures are only approximate since recovery was not checked for all amino acids. Recovery for valine, glycine, and phenylalanine, using C^{14}-labelled compounds, was 55.5%, 54.5%, and 55.5% at 5.0 micromoles per liter.

Oxygen consumption of these worms is of the order of 0.1 ml./gm./hr. If amino acids were the substrate, this is roughly equivalent to 0.1 mg. per hour. At the rate of uptake of glycine by *Clymenella* observed using glycine-C^{14}, the observed concentration of glycine alone in sea water would supply 30% of this amount. The other amino acids which have been studied (phenylalanine, valine, and lysine) are also accumulated by *Clymenella*. Although uptake from complex mixtures has not been investigated, it may be suggested that the free amino acids of its habitat offer this organism a significant nutritive source. Since *Clymenella* is often found in low oxygen environments, it is of interest to report that their uptake of amino acids occurs unimpaired in nitrogen-saturated water.

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Uptake of amino acids by the bamboo worm, Clymenella torquata. GROVER C. STEPHENS.

The observations to be summarized were made using C^{14}-labelled amino acids. Labelled phenylalanine, valine, glycine, and lysine were supplied in the ambient sea water. Entry of amino acids was followed by measuring the radioactivity of the sea water and of 80% ethanol extracts of the worms. The uptake of phenylalanine will be discussed as typical.

Uptake is linear with time for at least the initial stages of the process. After fifteen minutes at an ambient concentration of 10^{-6} to 5×10^{-5} molar, the radioactivity of an alcohol extract of the worms is approximately ten times that of the medium after correction for volume, self-absorption, and background. Chromatography indicates that the radioactivity of the extract is in the form of phenylalanine. Further increase in ambient concentration does not produce a corresponding increase in rate of uptake. The rate of uptake is a function of surface and occurs across the body wall, independent of the gut. There is some incorporation after 24 hours but the bulk of the amino acid taken in remains in the alcohol-soluble fraction. Once accumulated, amino acids are not exchangeable with amino acids in the medium to any significant extent. By pre-loading the organisms, accumulation can be observed against gradients of 5000:1 or greater. Uptake is not stereospecific, at least for phenylalanine. The Q_{10} for the process is approximately 1.7 for the temperature range 5–25° C.

Glycine is accumulated at approximately the same rate as phenylalanine. Lysine and valine enter at about 30% to 40% of this rate.

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Osmotic pressure relationships in the spiny dogfish (Squalus acanthias). WILLIAM STONE, JR. AND WILLIAM C. DEWEL.

Recent evidence (*Science*, **132**: 36, 1960) indicates that the osmotic pressure of the aqueous humor is lower than blood plasma in the smooth dogfish (*Mustelus canis*). However, other workers (*Comp. Biochem. Physiol.*, **5**: 193, 1962) have reported no difference in the osmotic pressure of these two fluids in the spiny dogfish (*Squalus acanthias*). The present study was undertaken to resolve the apparent discrepancy. The two species belong to different sub-orders, and differences in the morphology of the eye as well as the whole fish were numerous and pronounced. The osmolarity of the plasma and aqueous humor from both species were measured, using the Fiske osmometer. The results for the smooth dogfish were similar to those reported earlier. Extracting sufficient aqueous humor for measurement from the spiny dogfish was difficult but adequate samples were obtained from 10 large fish. The osmotic pressure of spiny dogfish aqueous humor was found to be 963 (S.D. 28.3) milliosmoles while the plasma measured 983 (S.D. 18.6). The average difference between the two sets of measurements was 20.6 milliosmoles. In only one case was the osmotic pressure of the aqueous humor higher than the plasma. In the remaining 9 cases it was lower. Statistical analysis, using the t test, revealed that the lower osmotic pressure in aqueous humor, as compared with plasma, was significant at 0.01 confidence limits.

Studies of melanin biosynthesis in the ink sac of the squid (Loligo pealei). II. (Histology, autoradiography, tissue culture and in vivo inhibition of ink gland). GEORGE SZABÓ AND R. T. SIMS.

The ink gland is unique in many ways as an experimental model for the study of melanogenesis. Not only does it produce large amounts of tyrosinase and melanin but it is apparently continuously synthesizing these materials.

There are two types of epithelial cells in the gland. (1) A columnar cell with well defined polarization, showing a strongly basophilic cytoplasm towards the basement membrane and large pigment granules towards the lumen. The granules were found to be melanin by histochemical tests. The nucleus is large with a thin chromatin network. There are several nucleoli. (2) The other type of epithelial cell is in the caudal portion of the gland. It is tall columnar, has little or no melanin, but contains a single large vacuole at the apical end. This vacuole does not contain PAS-positive material. The epithelium of the ink gland is tyrosine- and dopa-positive.

The rate of melanin production was studied by inhibiting tyrosinase activity with phenylthiourea added to sea water (0.004% and 0.008%). The ink gland starts to turn white after the living newly hatched squid has been in PTU for 48 hours. When returned to normal sea water they start to regain pigment after 24 hours. Autoradiographic studies of the gland of newly hatched squid showed uptake of H^3-tyrosine at a high rate and indicate a turnover of melanin of 24 hours or less.

The ink gland was cultured on glass surface in Gatenby's molluscan saline, in Hedon-Fleig saline with or without horse serum. The epithelial cells migrated either individually or in a sheet. The cells retained their polarity during migration, as the pigment was concentrated at one end. Movement of cilia was observed in the explant and isolated cells moved in the fashion of ciliated cells. Electron microscope pictures confirmed the existence of cilia.

Inhibition of regeneration in Tubularia by tissue extract injection. KENYON S. TWEEDELL.

Extracts of individual parts of adult hydranths were injected into the coenosarc cavity of amputated *Tubularia* stems. The tissues were homogenized in a small amount of filtered sea water with an iced Teflon homogenizer. The homogenates were then centrifuged at 30,000 *g* for 20 minutes in a refrigerated centrifuge. The supernatant was removed and refrigerated. Freshly amputated stems were injected from the proximal end with 0.5 to 1.0 μl. of extract, and then placed in standing filtered sea water at 19–21° C. Other stems were injected at 6, 12, 18 and 24 hours post-amputation. For the latter, a minute opening was made with a "00" needle at the healed distal end, to eliminate pressure. Controls were amputated untreated stems and amputated stems injected with filtered sea water.

Hydranths were subdivided into the distal hypostome, including distal tentacles, and the complete proximal hydranth. A single injection of the proximal hydranth extract (0.04 hydranth/μl.) up to 24 hours after amputation caused complete and permanent inhibition in 90% or more of the stems. Single injections of the distal hypostome with tentacles at 0, 6, or 12 hours after amputation retarded development in most stems for 24–30 hours. Thereafter, many retarded stems (from 40–100%) would recover and regenerate latently. Extracts injected at 18 hours or later had little effect.

Individual extracts were also made from distal hypostomes, gonophores, proximal tentacles and the remaining basal hydranth in concentrations from 0.03 to 0.05 part/μl. Each of the extracts was singly injected from 0 to 18 hours post-amputation (one band stage). The most effective tissue fractions were the basal hydranth and the gonophore extracts. Total inhibition of stems by the tissue extracts was not significant over the controls. Instead, development was often retarded up to 24 hours after injection. Alternatively, stunting of the hydranth occurred in 22 to 45% of the stems. This often reduced the number and length of the proximal tentacles. The extracts were effective at 0, 6 and 12 hours after amputation but had little action thereafter.

Analysis of motility in a new species of gregarine. CHRISTOPHER D. WATTERS.

The acephaline gregarine, *Urospora* sp. (after Torch), exhibits characteristic motions which may be observed through the body wall of its annelid host, *Pectinaria gouldii;* this coelomic parasite has continued such movements up to eight hours after isolation in filtered sea water.

The movements of specimens 80 to 500 microns long were recorded cinematographically for detailed analysis: (1) The most obvious movement is a wave of constriction that is propagated unidirectionally along the body axis from the more narrow end which attaches to the substratum; velocity of the wave is *ca.* 150 microns/second and the period about 2 seconds. (2) Cytoplasmic inclusions are observed to move rapidly in a direction opposite to that taken by the propagated wave, *until* the wave has passed midway along the body length; then, as the wave traverses the rest of the organism, these inclusions reverse their direction, first in the wave region, and thereby move more slowly with the wave. (3) The entire cytoplasm, including the nucleus, shuttles back and forth during the propagation of one wave. (4) When the surface is marked with carmine particles, it can be seen that each wave is accompanied by a 90° recoiling torsion of the whole organism. (5) Carmine particles on selected surface regions sometimes are swept to the ends of the cell. While this type of motion could cause the typical gregarine gliding movement, longitudinal displacements along a substratum were observed only when the organisms were compressed under a coverglass.

Observations with phase, interference, polarizing, and electron microscopes have so far revealed only a weak, generalized cortical birefringence (apparently *not* due to resolvable myonemes) and at the very surface spirally arranged rows of ridges of *ca.* 2 microns high; when the wave is propagated along the cell surface, these ridges move apart and then back together after the wave has passed. Further studies are in progress in collaboration with Delbert Philpott (electron microscope) and Robert Allen (polarizing microscope).

Seasonal fluctuations in mean paths of snails (Nassarius) in a uniform light field.
H. MARGUERITE WEBB AND FRANKLIN H. BARNWELL.

The mean paths taken by snails in a uniformly illuminated field were observed under the following conditions: in the morning, heading south and heading north, in the afternoon heading south and heading north. The test group in each case consisted of 10 animals which were observed for three trials in one direction and then three trials in the opposite direction. The observations were made during the period June 27 through August 24, 1962. When results were averaged, regardless of direction or time of day, and grouped into 7-day periods it was found that the mean paths varied throughout the study in the following manner: for the period beginning June 27 the path was 4.5° ± 0.5 to the left; for the period beginning July 4 the path was 0.2° ± 0.4 to the left; thereafter for five periods there was a progressive increase in amount of left-turning until the period beginning August 13 when the path was 8.6° ± 0.5 to the left. The intervening values were 4.7°, 5.9°, 6.2°, and 7.0°, all with comparable standard errors. For the period beginning August 20 the path was 1.7° ± 0.5 to the left. The coefficient of correlation ($r = 0.857 \pm 0.107$) between the paths of southbound and of northbound animals indicates that the two groups were similarly affected by whatever brought about the variation in mean paths.

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Studies on the structure of the thymus. I. Electron microscopic observations on the cortical vascular barrier. LEON WEISS.

The presence of a vascular barrier in the thymus, similar to the blood-brain barrier, has been postulated by Marshall and White because antigen injected intravenously induces no antibody response in the thymus, whereas antigen injected directly into the thymus does.

In the mouse, fine cortical vessels have an outside diameter of 4 to 8 μ and a lumenal diameter of 1 to 3 μ. Endothelial cells form a complete lining. Many lumenal processes are present and the cytoplasm is rich in vesicles. Basal endothelial processes extend into a broad basement membrane. Several layers of adventitial cells may be present. Each adventitial cell is surrounded by extracellular tissue consisting of ground substance and collagenous fibers, and continuous with the basement membrane. Thus, the vessel wall is often lamellated, layers of cytoplasm alternating with layers of extracellular tissue.

The most peripheral element in the wall is the epithelial reticular cell. It is a large cell whose cytoplasm may envelop the vessel. It may also extend cytoplasmic processes which surround perivascular lymphocytes. Reticular cells have desmosomes, show evidence of marked reduplications of the plasma membrane, and possess many vesicular processes. Reticular cells contain granules, suggesting secretion, and phagocytic vacuoles. Both granules and vacuoles are stained in the periodic acid-Schiff reaction. Reticular cells form Hassall's corpuscles.

After a single dose of Thorotrast, thorium dioxide is found primarily in the extracellular tissue of the vessel wall.

The morphological arrangement of these vessels is similar to that in the blood-brain barrier. They also resemble sheathed arteries in the spleen. It appears that thymic reticular cells mediate humoral influences upon perivascular lymphocytes.

The incorporation of iododeoxyuridine by the developing Arbacia embryo. M. B. WHEELER, C. V. HARDING, W. L. HUGHES AND W. L. WILSON.

Under certain conditions, iododeoxyuridine (IUDR), an analogue of thymidine, can be incorporated into DNA. There is evidence, furthermore, that its incorporation mimics that of thymidine (Prusoff, 1960; Gitlin, 1961). When labeled with I^{131}, the uptake of IUDR can be detected externally with a scintillation counter. The possibility exists, therefore, that an index of the rate of DNA synthesis can be determined in the same tissue or group of cells at several different times. As a preliminary to such determinations, the present study was conducted to determine the extent of uptake of IUDR into the *Arbacia punctulata* embryo at different stages of development. Experiments were performed, in each of which the eggs from a single female were used; aliquots of the developing embryos were taken at various times after fertilization, incubation in IUDR (carrier-free, 0.022–0.006 μC./ml.) for periods of 0.5 to 1.0 hour, and then their radioactive content was determined. The concentration of IUDR and the duration of incubation were maintained constant for the aliquots within a given experiment. After incubation, the eggs were washed 3–4 times in sea water and extracted three times with cold 5% TCA or Carnoy's solution. Radioactivity in the sea water washes and in the acid-soluble and -insoluble fractions was then determined. There was insignificant incorporation into the acid-insoluble fraction and very little into the acid-soluble fraction of the unfertilized eggs. Following fertilization, there was a significant incorporation into both fractions. The rate of incorporation increased with increasing age of development until at the end of 15 to 18 hours, the curve reached a maximum, and decreased thereafter for several hours. The radioactive component(s) in the acid-soluble fraction was apparently not in the form of IUDR since the radioactivity did not crystallize with added carrier IUDR. The incorporation of IUDR into both fractions was strikingly inhibited in the presence of thymidine (conc., 0.002 M).

Studies on Euplotes. I. Structure and life cycle of a new species of marine Euplotes. RALPH WICHTERMAN.

Characteristics: Body 132 × 70 μ, somewhat convex dorsally and ventrally with 11 laterodorsal kineties. Anterior end widest and truncated; posterior end rounded. The adoral zone of membranelles extends to three-fourths the body length. Cirri: 10 frontal-ventral; 5 anal; 4 caudal. Macronucleus 82 μ, band-like and C-shaped. Micronucleus 3 μ. Location: Bay of Naples.

From collections, isolations were made and 6 clonal cultures established in filtered sea water with associated bacteria, to which was added Cerophyl, a powder of dehydrated cereal grass leaves. At the log-growth phase, the fission rate averaged 1.75 divisions per day. Fission required one and a quarter to one and a half hours, from the time the vegetative animal was seen to begin division until the two separated daughters were formed. Mean salinity of sea water of the Bay of Naples is 38. Organisms are presently being cultivated and studied in sea water from Woods Hole Harbor, which has a salinity of 31–32.

Attempts were made to determine the existence of mating types for a genetic analysis by making all possible mixtures of clones. Mating and conjugation did not occur. However, the processes of encystment and excystment took place and could be controlled.

Normally, in an old culture, animals encyst. For this to occur, the vegetative animal stops eating, settles to the bottom of the container and undergoes dedifferentiation of cirri and membranelles. Seen from top view, cysts appear circular and average 78 μ in diameter. Seen from side view, the upper surface of the cyst is highly convex but the lower surface only slightly convex. A sticky substance secreted by the encysting animal enables the cyst to adhere to the substratum.

In a rich culture, encystment can be induced by sharply cutting off the supply of available food. Excystment can be accomplished by surrounding the cysts with fresh medium. When single cysts were placed in this medium, no excystment occurred for at least one and a half hours, but most animals excysted not later than 5 hours, after which they resumed normal fission.

Part of a project aided by grants from the American Philosophical Society, the Committee on Research of Temple University, and the American Tables Committee for the Naples Zoological Station.

Studies on Euplotes. II. Mating types and conjugation in a marine species of Euplotes. RALPH WICHTERMAN.

Characteristics: Body ovoid, 50 × 27 μ, slightly convex dorsally and ventrally with 8 laterodorsal kineties. Adoral zone of membranelles extends to four-fifths body length. Cirri: 10 frontal-ventral; 5 anal; 4 caudal. Macronucleus 26 μ, band-like and C-shaped, with posterior end consisting of a small knob attached to larger part by a thin strand. Micronucleus 1.5 μ. Location: Bay of Naples. Investigations at present reveal that the ciliate may be *Euplotes cristatus* or a closely related species, if not a new one.

Nine clones, designated A, G, H, J, K, L, M, N, and Q are in cultivation in filtered sea water from Woods Hole Harbor. The results of all possible mixtures of the nine clones revealed the existence of mating types as follows: clone G mated with all clones except A and N; clones H, J, L, and Q mated with all clones except A; clone K mated with all clones except A and M; clone M mated with all clones except A and K; clone N mated with all clones except A and G. Obviously, clone A failed to mate with any other clone.

Upon mixing reactive opposite mating types, two ciliates ready to mate spiral rapidly forward and parallel to each other on a longitudinal axis. After this cooperative spiralling, animals join along their adoral zone of membranelles. They do not join in the sexual union immediately after being mixed but from 3 to 12 hours later at 26° C. Data thus far suggest they mate more readily in the morning, beginning with daylight, than at other times. Mating pairs remain joined for approximately 20 hours.

In the sexual process the macronucleus of each member segments into three, then four, lobes, each connected by a thin strand of nuclear material. After the strands break, two of the lobes become localized at the anterior end of a conjugant and two near the posterior end. Those in the posterior end are the first to disintegrate.

Concomitant with macronuclear breakdown the micronucleus of each conjugant undergoes the pregamic divisions. A single spherical anlage is produced in each, which later occupies about one-third the body length. In Feulgen preparations the anlage appears homogeneous and does not react to the stain. As long as 80 hours after mating, the anlage is then faintly Feulgen-positive while the micronucleus adjacent to it is well stained.

Part of a project aided by grants from the American Philosophical Society, the Committee on Research of Temple University, and the American Tables Committee for the Naples Zoological Station.

A comparison of methods using Ca⁴⁵ as a tracer for calcium activity in Arbacia eggs. FLOYD J. WIERCINSKI AND CAROL E. WIERCINSKI.

During the past four summers Ca^{45}, in combination with various methods, has been used to determine whether or not calcium ion is absorbed or released in the eggs of *Arbacia punctulata* before and after fertilization. A Geiger tube and a gas flow β detector with dried planchet samples of eggs yielded results in the range of the standard deviation. Autoradiographic study of sectioned ovary from *in vivo* experiments with Ca^{45} indicated the presence of the radioisotope in and around the immature cells. Studies of sectioned eggs before and after fertilization indicated Ca^{45} in the jelly coat and on the cortical layer. Filtration of incubated eggs showed significant amounts of activity related to the mass of cells after fertilization.

Arbacia and *Laminaria* were put into aerated Ca^{45} sea water for a period of 6–10 days. The eggs of these animals were shed into 150 cc. of fresh sea water by means of electrical stimulation and were found to be moderately radioactive. A Geiger tube placed above a dish of settled or agitated eggs made no significant difference in the count. A Geiger tube was placed into the sea water 0.5 mm. above the layer of eggs. Also, a glass cylinder was placed over an inverted tube so that the window formed the bottom of the vessel with eggs resting on the window. Liquid scintillation counting techniques were used with samples taken from 1 cm. of sea water above the egg layer resting at the bottom of a beaker. Statistically significant counting showed no difference of Ca^{45} activity before and 60 minutes after fertilization. The above experimental conditions and methods give data to indicate that calcium does not move and is neither absorbed nor released on fertilization. Other experiments are planned.

The growth of brain in teleosts. CHARLES G. WILBER AND RICHARD SCHNEIDER.

The expression of relative growth of organs with respect to growth of total body is usually formulated in logarithmic terms. The log-log relationship leads to calculation of "constants of allometry" and other constants, some of which are biologically meaningful. Nevertheless logarithmic relationships are not particularly easy to visualize, especially by the non-mathematician. It is our contention that, for certain aspects of relative growth, the rectangular hyperbola is especially useful because such a curve is easily visualized and presents a generalization which makes sense biologically over a wide range of values. Measurements of brain weight and total body weight were made for a number of local marine species of bony fish. The results were plotted on ordinary coordinate paper. Plotted points fell along a path which by inspection described an hyperbola. Curves were fitted to the points by successive approximation. Examples of the fitted curves are given in the following expressions: for the sea bass, *Centropristes striatus*, brain weight in mg. equals 420 minus the quantity 32,000 divided by body weight in grams; for the puffer, *Sphaeroides maculatus*, brain weight in mg. equals 380 minus the quantity 10,800 divided by the body weight in gm.; for the scup, *Stenotomus versicolor*, brain weight in mg. equals 807 minus the quantity 61,500 divided by body weight in gm. Growth of brain in tautog, sea robin, and flounder seems to follow the same general pattern. Detailed analyses of the relative growth of the following organs (in addition to brain) with respect to body growth are being made: eyes, liver, gut, spleen, heart.

Observations on marine eggs subjected to ultrasonic vibration. WALTER L. WILSON, FLOYD J. WIERCINSKI, WESLEY L. NYBORG AND F. J. SICHEL.

Eggs were subjected to ultrasonic vibration by means of a steel needle applied directly to the cell surface or inserted into a drop of an egg suspension. The needle was mounted in the tip of a steel cone fixed at its base to one end of a cylindrical, ceramic transducer. The transducer was driven by means of an oscillator working through a power amplifier and had a tuned frequency of about 83,000 cycles per second. The needle, with a shaft diameter of 0.2 mm., extended about 3 mm. beyond the tip of the cone and tapered to a blunted tip. Careful machining and mounting ensured that the vibrations induced in the needle were in the long axis, with little lateral motion. In some experiments eggs were held stationary on the end of a micropipette; in other experiments eggs were allowed to move freely in a drop of sea water. Observations were carried out with the aid of an inverted microscope, and motion pictures were taken.

With the needle tip applied directly to the cell surface of unfertilized eggs of *Asterias* or of *Spisula* held with a micropipette, ultrasonic vibration in some cases causes the nucleolus to move about within the nucleus; in other cases the nucleolus remains in a given position but rotates, turning faster as the intensity of the ultrasonic energy is increased. In one *Asterias* egg the nucleolus was observed to break into two parts.

Unfertilized eggs of *Asterias* free to move in a sea water drop move close to the vibrating needle. In this position the surface of the egg closest to the needle undergoes undulations. In several eggs rather large cone-like projections were formed at the cell surface.

This work was supported by a Grant (RG-8775) from the National Institutes of Health.

DNA synthesis in early mitotic stages: a pressure study. ARTHUR M. ZIMMERMAN.

Fertilized eggs of *Arbacia punctulata* were placed into tritiated thymidine (1–2 μC./ml.) at early stages of the first mitotic cycle. Immediately after immersion in the isotope, the fertilized cells were subjected to hydrostatic pressure for varying periods of time. The pressure chosen, 5000 lbs/in.^{2}, has previously been shown to block the formation of the mitotic apparatus as well as the furrowing reaction in cleaving eggs; in eggs with well-formed spindles and asters this pressure causes drastic disorganization to the mitotic apparatus. Following pressure treatment, some of the cells were permitted to develop and some were placed into fixative. The paraffin-imbedded material was sectioned and subjected to autoradiography. Alternate slides were subjected to DNase digestion prior to autoradiography. The incorporation of tritiated thymidine was employed as an index of DNA synthesis.

When pressure treatment was initiated at presyngamy (5 minutes after insemination) and maintained for 60 minutes, incorporation of H^3-thymidine into nuclear DNA was established in both male and female pronuclei prior to their union. Evidently, this pressure-temperature treatment (5000 lbs/in.^{2} at 20° C.) blocks the union of the pronuclei, which normally occurs within 12 minutes after insemination. The incorporation of H^3-thymidine is localized in the pronuclei. Moreover, when the pressure treatment is initiated after syngamy (15 minutes after insemination) the eggs incorporate H^3-thymidine in the zygote nucleus.

The data presented indicate that pressure as high as 5000 lbs/in.^{2}, which may cause extensive cytoplasmic disorganization, does not block DNA synthesis. Furthermore, the incorporation of H^3-thymidine into chromosomal DNA may occur prior to or after syngamy.

Work supported by grant GM 07157-03 from the Division of General Medical Sciences, U. S. Public Health Service.

The effects of mercaptoethanol on cleaving eggs of Arbacia punctulata. ARTHUR M. ZIMMERMAN.

The fertilized eggs of *Arbacia punctulata* were immersed into various concentrations of mercaptoethanol, and the structural state of the cortical cytoplasm, as well as the "cleavage potential" of the cells were measured. Previous studies have established that mercaptoethanol has a marked effect on the mitotic apparatus. Mercaptoethanol blocks the formation as well as disorganizes the structural integrity of this highly complex structure.

Pressure-centrifuge measurements of the structural state of the cortical cytoplasm were made at various pressures (6000–12,000 lbs/in.^{2}) at 20° C., employing a centrifugal force of 33,000 *g*. Immersion treatment in mercaptoethanol was initiated 20 minutes after insemination. After 20 minutes' incubation, the eggs were subjected to pressure-centrifugation. Two concentrations of mercaptoethanol were employed. A blocking concentration of mercaptoethanol, 0.075 *M*, yielded a value for the strength of the cortical gel which was 22–24% lower than that found in the non-treated controls. At a lower mercaptoethanol concentration, 0.01 *M*, division was not blocked and the gel strength curve was parallel to the curve for the blocking concentration and the control curve, but lying intermediate between the two.

The decrease in the gel strength was shown to be related to a decrease in the "cleavage potential." A pressure of 4500 lbs/in.^{2} applied at the time of furrowing will, in general, block about 50% of the cells from cleaving. When the eggs were pretreated with 0.01 *M* mercaptoethanol 20 minutes prior to division, there was a 24% lowering in the number of cells which completed division under pressure treatment as compared to the non-treated pressurized controls.

In general, the data support the hypothesis that interference with the $SH \rightleftarrows S\text{-}S$ interaction in protoplasmic gel system is similar in both the mitotic gel system and the cortical gel system and any interference with the delicate balance may markedly disrupt mitosis and cytokinesis.

Work supported by grant GM 07157-03 from the Division of General Medical Sciences, U. S. Public Health Service.

LALOR FELLOWSHIP REPORTS

Blood protein changes in Crustacea. HANS LAUFER AND THOMAS McNAMARA.

The hemolymph of *Uca pugnax* and *Uca pugilator,* decapod Crustacea, was examined by zone electrophoresis in starch gels in intermolt, during normal molting, and after the initiation of molting induced by eyestalk ablation. Histochemical staining of gels revealed serum proteins, hemocyanins, and esterases. The purpose was to determine whether there are changes in the patterns during molting.

About 300 *Uca* were studied, of which more than 50 molted in the laboratory. In one experiment, 50% of the eyestalkless animals and 20% of the controls molted during the first month. The protein concentration was followed over a period of five days in the same individuals after eyestalk removal. The average protein content, as measured by the Biuret reaction, before the operation was approximately 21 mg./ml. (16 animals). The controls lost approximately 4 mg./ml. after five days; the experimentals showed a decrease of more than twice this amount. Newly molted *Uca* (6) averaged the same low concentration as the eyestalkless animals.

Analysis for serum proteins, esterases, and hemocyanins in starch gels revealed the following: Two major blood proteins are present in all control and experimental animals, with no consistent differences between species. A third minor component was observed in some of the experimentals. The esterases of controls are most commonly either two or three (75%). Experimentals had fewer esterases. One hemocyanin band is detected in 70% of the control animals, two bands in 30%. Only one hemocyanin band was displayed by 93% of the experimentals. The hemocyanins, therefore, exist in two forms which often occur together in control animals, but are rarely together in experimentals. Thus, contrary to the recent report of Woods *et al.* (1958), proteins change during molting in quantity, and in diversity. Considerable individual differences in the changes of blood proteins during molting suggest that these changes are influenced by additional, yet uncontrolled variables.

Supported in part by grants from the N.S.F. and the Lalor Foundation.

Intracellular pH in Arbacia eggs. ROBERT W. WINTERS.

The distribution of the weak acid 5,5-dimethyl-2,4-oxazolidinedione (DMO) has been studied in unfertilized *Arbacia* eggs in order to calculate the "aggregate" intracellular pH. This method is based on the principle that if the total concentration of DMO per unit volume of egg water is known, "aggregate" intracellular pH can be calculated on the reasonable assumption that the undissociated moiety of the compound achieves equal concentrations on both sides of the membrane. A spectrophotometric method of analysis for DMO, based upon the finding of an absorption peak at 208 mμ, was found to be unsatisfactory because of interfering substances. A radioactive method was therefore used employing C^{14}-DMO. Binding of DMO by intracellular components was excluded in prolonged dialysis experiments using egg homogenates. Recovery experiments demonstrated that over 96% of added counts could be recovered in a form showing identical behavior to DMO, with respect to partition between ether and aqueous phases, as pH of the aqueous phase was altered. DMO in concentrations up to 4 m*M* did not interfere with normal fertilization or early cleavage. The major uncertainty of the method is the volume to be assigned to the trapped medium within the centrifuged pellet. Studies of this volume using I^{131}-albumin, C^{14}-carboxyinulin and C^{14}-sucrose demonstrate that the former may give artifactually high values under certain conditions. Using either of the latter substances, the calculated intracellular pH was found to be between 6.5 and 6.8 and to be relatively independent of wide shifts in ambient pH (4.5 to 8.5) produced by addition of strong acid or base.

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OBSERVATIONS ON BURROWING IN THE VENERIDAE (EULAMELLIBRANCHIA)

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Among bivalves, the conflicting requirements of maintaining contact with the surface for feeding, and retiring into the substratum to avoid disturbance or for protection against enemies have led to adaptations of the foot and associated muscles, and the siphons in a number of different ways. In shallow-burrowing bivalves, mobility is essential, and such animals possess a large wedge-shaped foot capable of being protruded at any angle ventrally through a large pedal gape. Such active shallow-burrowing animals represent a relatively unspecialized type within the bivalves, and it was probably from similar types that the more specialized deep-burrowing forms evolved, although by many separate, convergent and divergent routes.

The result of such evolution along separate lines may be seen in the sub-order Veneracea: members of the Petricolidae are adapted to a greater or lesser extent to a rock-boring habit (Purchon, 1955; Yonge, 1958). In the Glaucomyidae mobility has been lost and the animal is embedded permanently in the substratum at a considerable depth (Owen, 1959). The genus *Venerupis* of the Veneridae shows affinities with both these groups and with the shallow-burrowing genera such as *Gafrarium* and *Venus* (Ansell, 1961). The genus *Dosinia* has taken a course to deeper burrowing: the shell is almost circular and lies normally with the ligament more or less parallel to the surface, the powerful foot is protruded ventrally and the anterior and posterior sets of retractor muscles perform equal work in the digging process, and the flattened lunule area anterior to the umbones possibly assists burrowing by acting as a pressure plate preventing the animal from moving upwards as the foot is extended. This paper describes observations of the burrowing movements of some members of the Veneridae and attempts to define a generalized time/motion pattern for this type of activity.

Apart from brief references in more general papers, the literature on the digging movements of bivalves is scanty. Locomotion in members of the Protobranchia has been described by Drew (1899), Vlès (1904), Morse (1913) and Stoll (1938). Methods of locomotion in various other species have been described

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by Drew (1907), Jordan (1915) and Stoll (1937, 1938), but the only papers of real significance are those of Fraenkel (1927), which gives a fairly comprehensive description of co-ordination of movement in the Solenidae, and of Quayle (1949), describing movements in *Venerupis* (= *Paphia*) *pullastra.*

Material and Methods

Recordings were made of the time sequence of movements of *Venus striatula, Venus casina, Dosinia lupinus, Venerupis decussata* and *Mercenaria mercenaria.* Some of these were made with the animal attached to a lever system writing on a kymograph (Fig. 1). In others the time sequence only was recorded manually using a stop watch to time the intervals. In the latter cases it was found possible to continue recording even after the animal was completely buried, by observing the characteristic sequences of opening and closing of the siphonal apertures and extension of the siphons described later. All the observations recorded here were made with the animals burrowing in clean sand; for *Venus striatula, Dosinia lupinus* and *Venus casinia* from Kames Bay, Millport, Scotland, for *Venerupis decussata* from Hamworthy Beach, Poole Harbour, England, and for *Mercenaria mercenaria* from the shores of Pivers Island, Beaufort, North Carolina, U.S.A. No attempt was made to use substrata from the normal habitat of the species. No grade analyses were made but the sands were apparently of similar constitution.

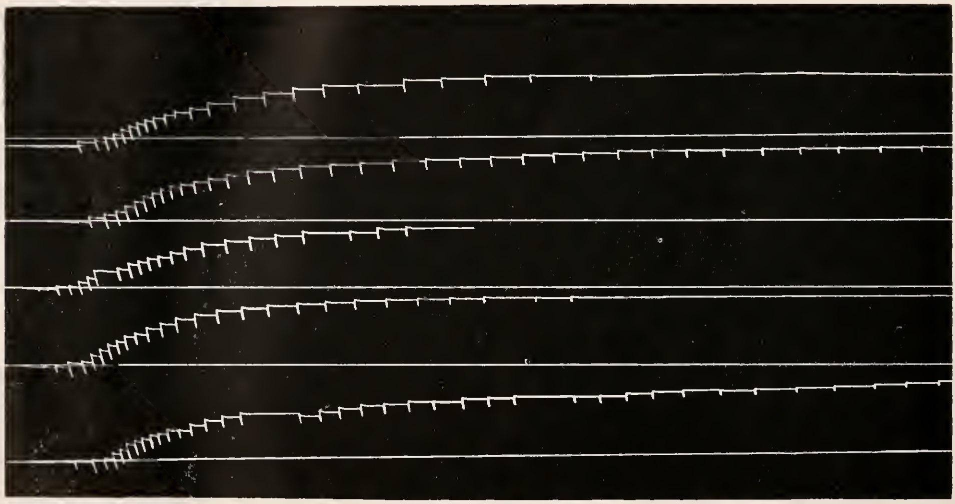

Figure 1. Kymograph records of five consecutive digging-periods for *Mercenaria mercenaria.*

The Normal Series of Burrowing Movements

In describing the movements performed in burrowing in the Solenidae, Fraenkel (1927) used the terms "Grabstufe" and "Grabperiode," English equivalents of which may be given as "digging-sequence" (Quayle, 1949) and digging-period. These two terms in the English form will be used here in their original meanings, the digging-period being the period from the start of burrowing until the final position is reached, and the digging-sequence one of the number of separate downward movements of which the digging-period consists.

Quayle (1949, pp. 32–33) described the digging-sequence in *Venerupis pullastra* as follows:

> "(1) The valves separate.
>
> "(2) The foot is protruded, pointed with a probing motion. This back and forth searching motion is continued until the foot is fully extended. The tip of the foot may extend to a length equal to that of the animal. The degree of vertical penetration in this phase may vary considerably and is partly dependent on the type of substratum.
>
> "(3) The heel of the foot is protruded vertically.
>
> "(4) The heel expands both laterally and posteriorly so that, coupled with the anterior extension of the foot, an anchor is formed. If the substratum is firm enough, the foot maintains this position and the shell moves.
>
> "(5) The valves open slightly.
>
> "(6) The two siphonal apertures close, and then the adductor muscles contract, reducing the volume of the mantle cavity, the excess water being forced out in a stream from the anterior end just below the adductor muscle. Presumably the pallial curtain (velum) maintains a seal around the foot and the remainder of the mantle edge during the operation. Almost immediately the anterior pedal retractors contract and the posterior retractors relax, causing the anterior end of the shell to dip and the posterior end to rise. It is probable, and this description of the play of the muscles is only conjectural, that contraction of the anterior retractors is confined to that part near its insertion into the shell.
>
> "(7) The final movement now takes place as the shell moves forward and down. The foot remains in its position of anchorage and the more distal portion of the anterior retractors now comes into action and the body is drawn forward. At the same time elements of the posterior retractors running downward and forward to the base of the foot contract, so assisting in the movement."

This description of the digging-sequence in *Venerupis pullastra* describes accurately the events occurring in the other members of the Veneridae studied. A few further points may be added, however.

At all times during the sequence and for the whole of the digging-period the tips of the siphons maintain contact with the surface of the substratum. If such contact is lost, through withdrawal due to disturbance or from some other cause, there is a break in the sequence of movements. During the digging-sequence the siphons perform a characteristic series of movements. Quayle (1949) has described the closing of the siphonal apertures, which occurs immediately before the final downward movement of the bivalve after anchorage has been secured, and which is associated with a jet of water being forced from the mantle cavity. After the completion of the downward movement, the siphonal apertures reopen. A short time later, if the shell is completely buried, the apertures close once more and the siphons are slightly withdrawn and then "stretched." The apertures then reopen.

The actual downward movement in each digging-sequence is brought about by a complex coordination of all the muscular systems of the animal. A detailed analysis of the role of each group has not been attempted. In general the active downward movement is brought about by the contraction of the anterior and posterior pairs of pedal retractor muscles, and is aided by the liquefying effect on the substratum of the jet of water ejected from the mantle cavity. Relaxation of the retractor muscles, as well as the changes in shape of the foot associated with anchorage, are brought about by means of the intrinsic musculature of the foot and visceral mass acting on a hydroskeleton provided by the blood filling the large sinuses.

The downward movement of all members of the Veneridae examined has associated with it a forward movement, with the result that the animal moves obliquely downwards. This forward movement is the result of asymmetry in the protrusion of the foot, the posterior end being anchored more or less immediately below the posterior margin of the shell by the heel, while the anterior end of the foot is thrust forward for some distance. In those bivalves where the protrusion of the foot is symmetrical no such forward component is present and downwards movement is vertical. Such vertical burrowing, by an exactly similar method to that described here, is seen in the case of some members of the Lucinacea—the Thyasiridae and Ungulinidae—where the heel of the foot is poorly or not at all developed (Allen, 1958).

Should the downward component on the shell be prevented from acting, the forward component results in horizontal movements. This is the case in adult bivalves moving on a hard substratum (Quayle, 1949, and personal observation). The essential similarity of the muscular action involved is indicated by the rocking movement of the shell which occurs in both cases. Essentially the same actions are responsible for the horizontal movements of young post-larval bivalves on hard substrata. The characteristic burrowing movements appear in the pediveliger stage (Ansell, 1962), although for some time the forward movement and the extension of the foot are aided by the beating of the strong pedal cilia. Such ciliary-aided movement may be retained in the adult stage of some bivalves, *e.g. Kelliella* (Clausen, 1958).

The Digging-Period

The digging-period constitutes the time between the initiation of burrowing and the attainment of the final position of the substratum, and consists of continued repetition of the characteristic digging-sequence. Although all digging-sequences are similar, the time taken to complete individual sequences, within the period, varies. The variation in time/sequence presents a characteristic appearance on analysis. Thus, if the time/sequence is plotted graphically against the number of that sequence in the period analysis curves of the type shown in Figures 2, 3, 4 and 5 are obtained.

Variations in the time/sequence are the result largely of variation in the time taken for the foot to obtain anchorage in stages (2) to (4) of the digging-sequence. In the early sequences of the digging-period this time is long in comparison to that of later sequences. These early sequences may be regarded as comprising an initial stabilizing period during which the movements of the foot serve to bring the shell into a vertical position where it is supported by the surrounding substratum.

Further sequences of movements follow rapidly at more or less equal time intervals. This period, during which the time/sequence remains more or less constant, ends when the shell reaches a position where the hinge margin is level with the surface of the substratum. Up to this time only the first siphonal movements of the digging sequence have occurred. The second series of siphonal movements described earlier are included in subsequent sequences, and these later sequences occupy progressively longer time intervals (Fig. 2).

The time pattern of repetition of sequences during the digging period was repeated in whole or in part by all members of the Veneridae examined. The fullest records were obtained with those animals which burrow more deeply, where the number of sequences making up the digging-period was greater. Deeper burrowing is apparently achieved by quicker repetition of the characteristic sequences and an increase in the number of movement sequences/period, and is associated with large size (*Mercenaria*) or with the possession of elongated siphons (*Dosinia*). In those species such as *Venus casina*, with short siphons, where the normal habit is to lie near the surface of the substratum with the posterior end of the shell exposed, the burrowing-period consists of the initial fixation and downward movements only and ceases when the ligament margin is more or less level with the surface of the substratum.

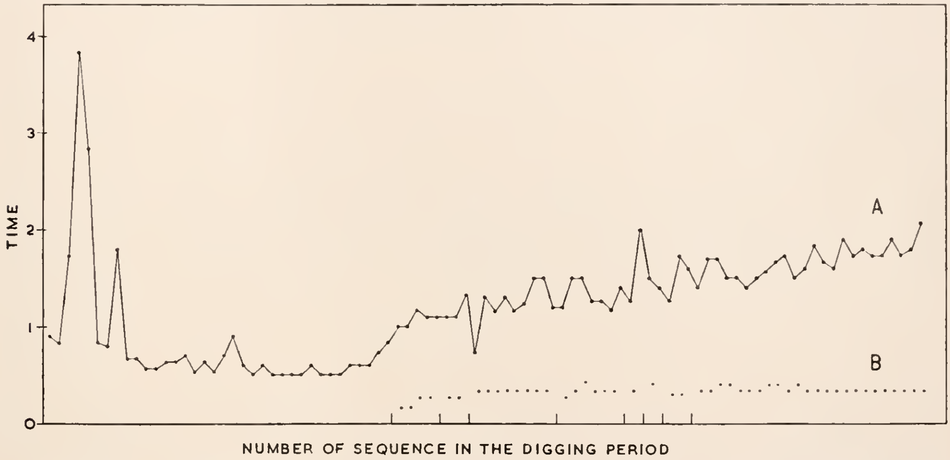

FIGURE 2. Analysis curves of time/sequence (mins.) for one complete digging-period for *Venerupis decussata* (A). The time interval between the final downward movement and the second extension of the siphons (see text) is also shown for each sequence (B).

If the change-in-depth/sequence is analyzed in the same way, a similar although opposite pattern appears. Depth/sequence falls off progressively as the time/sequence increases (Fig. 3). The early fixation sequences (not well represented in Figure 3) result in little change in the depth reached.

Analysis curves of records of the digging-periods of 17 *Mercenaria mercenaria* burrowing in sand are presented in Figure 4. Although there is considerable individual variation, the pattern for each animal is fairly constant and repeatable while the general overall pattern is discernible in each record. The most constant feature of comparison between repeated records from individual animals and

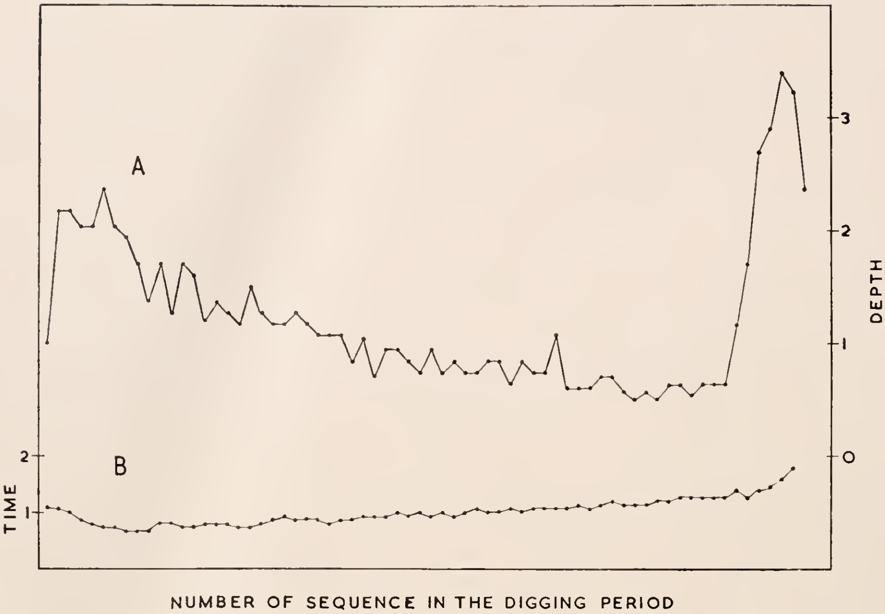

Figure 3. Analysis curves of (A) depth/sequence (mm.) and (B) time/sequence (mins.) for one complete digging-period for *Dosinia lupinus.*

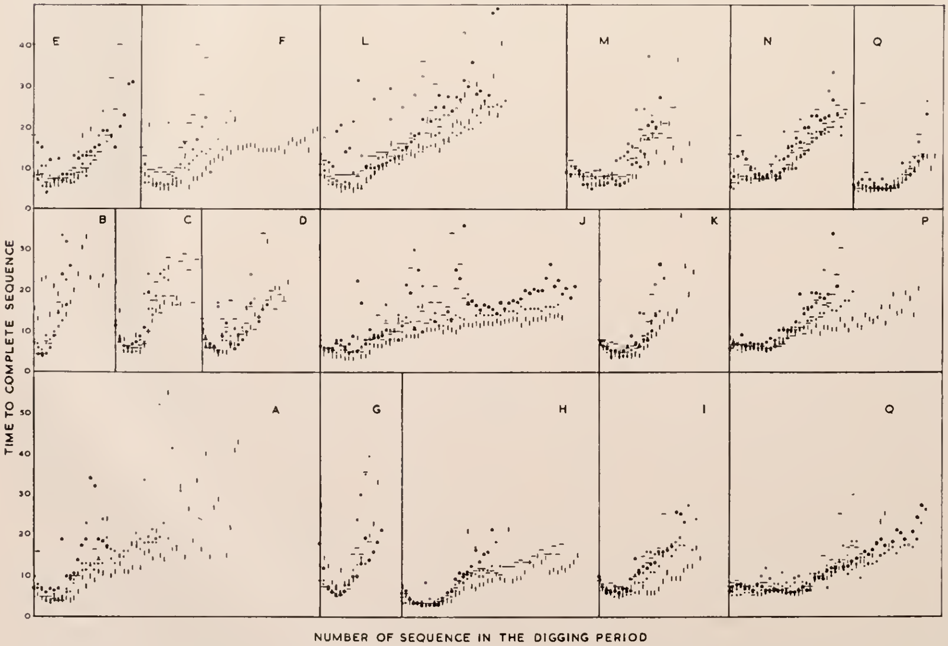

Figure 4. Analysis curves of time/sequence (mins.) for five consecutive complete digging-periods for each of 17 *Mercenaria mercenaria.*

between animals is the time/sequence in the period immediately following fixation, values for which are presented in Table I.

The total depth reached at the completion of burrowing in 84 cases from 17 *Mercenaria mercenaria*, together with the number of consecutive movement sequences/period, are also shown in Table I. The depth to which any individual animal will repeatedly burrow is relatively constant, and this is reflected to some extent in the number of consecutive sequences/period.

To record these observations the animals were attached to a kymograph lever by means of a thread fastened to the midposterior region of the shell. The depth recorded in Table I is thus the depth of this attachment point below the surface.

TABLE I

Time/sequence during the period immediately following fixation (see text), total depth reached and the number of consecutive sequences/period for each of five consecutive complete digging-periods for Mercenaria mercenaria *burrowing in sand*

Animal No.	Length (cm.)	Time/sequence (min.)					Total depth reached (cm.)					No. of consecutive sequences				
		1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
A	3.76	4.0	4.0	3.5	4.0	6.0	5.0	4.1	4.2	2.4	3.6	53	33	38	18	20
B	3.43	13.0	5.5	4.5	4.5	4.0	7.2	3.1	3.2	3.3	3.0	20	10	10	8	11
C	3.66	5.0	5.0	5.0	6.0	6.5	5.0	3.4	3.6	4.0	3.4	19	16	14	22	10
D	3.46	4.5	5.5	6.5	5.5	5.0	2.8	2.4	2.6	3.3	3.0	24	13	15	22	18
E	3.56	6.0	6.5	5.5	6.5	8.5	1.4	2.3	2.4	2.2	3.8	17	22	16	24	27
F	3.54	5.0	5.5	6.0	8.5	—	3.1	2.3	2.2	2.2	—	45	24	19	18	—
G	3.54	7.5	6.5	6.0	5.0	5.5	3.3	3.3	3.1	3.3	3.4	15	14	13	14	17
H	6.55	3.0	3.0	3.0	3.5	3.0	5.4	4.9	4.2	5.3	4.4	45	26	22	41	24
I	6.55	5.0	5.0	5.5	6.5	6.0	3.5	3.9	4.1	3.6	3.9	37	36	33	28	32
J	6.30	3.5	4.5	5.0	5.0	6.0	9.2	8.6	6.3	6.5	7.6	62	60	22	38	64
K	6.34	4.0	3.5	4.0	5.0	5.0	4.3	4.0	4.0	3.2	3.9	25	18	16	15	17
L	5.84	4.5	5.0	6.0	7.5	8.5	5.9	5.8	6.8	5.5	5.4	46	47	47	32	46
M	9.37	5.5	7.0	8.0	8.0	5.0	3.7	3.6	3.7	3.4	3.7	32	24	22	28	25
N	9.62	6.5	6.5	5.0	6.5	5.5	5.1	6.6	7.8	7.6	3.4	19	30	29	30	26
O	9.58	5.5	5.5	7.0	6.0	6.0	3.1	3.5	2.7	4.9	5.2	41	35	50	34	49
P	9.65	6.0	5.0	5.5	6.0	6.5	7.0	6.2	5.8	5.0	5.0	48	32	25	29	28
Q	8.50	4.5	4.5	5.0	4.5	5.5	4.6	3.9	4.4	3.6	3.7	22	20	18	19	20

At the completion of burrowing, most of the animals were orientated with the hinge margin more or less parallel to the surface of the substratum, and were completely buried.

In animals attempting to burrow in a hard substratum the time/sequence is irregular since the foot does not readily secure anchorage. A typical example of an analysis curve under such circumstances is shown for *Dosinia lupinus* in Figure 5. Also in Figure 5 is shown an analysis of the digging-period of the same animal in deep sand and in shallow sand over a hard bottom. The change in time/sequence as the animal reaches hard substratum from soft is readily seen.

DISCUSSION

The observations made here extend those of other workers and confirm that there is a basic sequence of movements involved in burrowing in bivalves. This basic sequence is common to all the Veneridae examined, and is similar to that described by Fraenkel (1927) for the Solenidae. Personal observation and scattered references in the literature suggest also that the movements in other groups conform to this pattern.

The characteristic pattern of time/sequence and depth/sequence may arise from the intrinsic nervous mechanism controlling the burrowing process, or from extrinsic environmental factors. These alternatives may be briefly discussed although further work is needed before the significance of this pattern can be fully appreciated.

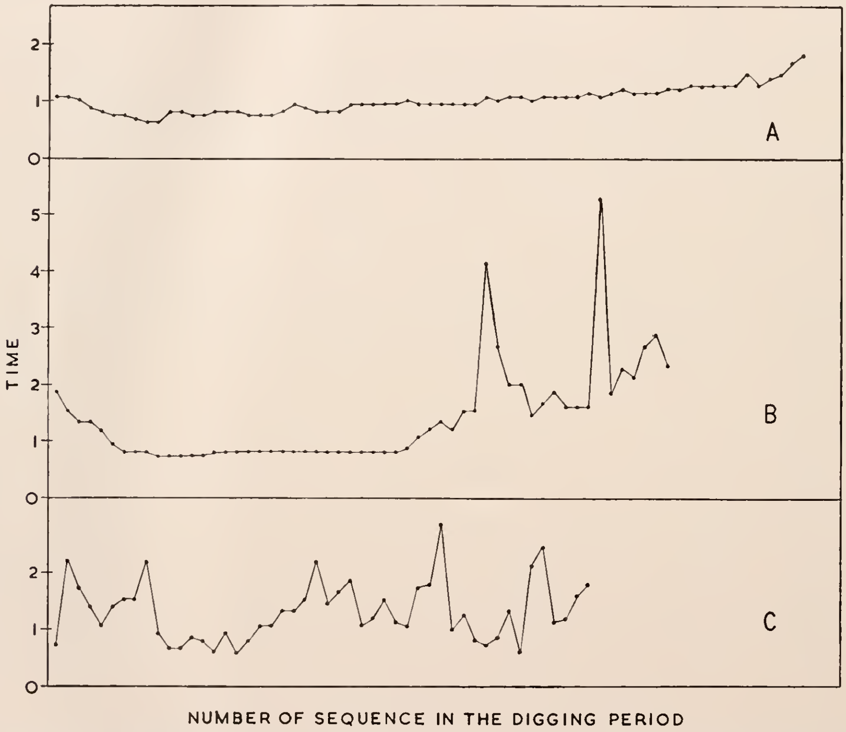

FIGURE 5. Analysis curves of time/sequence (mins.) for complete digging-periods for *Dosinia lupinus* (A) in sand, (B) in shallow sand over glass, and (C) on glass.

The gradual increase in the time/sequence and the decrease in change-of-depth/sequence noted may arise from a gradual increase in resistance to burrowing experienced as the animal moves deeper into the substratum. Since the greater time/sequence involves mainly an increase in the time for the foot to obtain anchorage in stages 2–4 this increase would suggest that penetration of the foot into the substratum becomes progressively more difficult, while the decrease in depth/sequence suggests that the substratum offers increasing resistance to penetration of the shell.

As an alternative explanation we may consider the possibility that the changes in time/sequence and depth/sequence indicate a change in the intensity of the burrowing response, *i.e.,* the digging-sequence, rather than the effect of a changing environment on a constant response. The observed changes would then indicate a gradual weakening of the response throughout the digging-period. Such a weakening could result from a gradual lessening of intensity of the burrowing stimulus, or alternatively an increase in some inhibitory stimulus opposing the response to the original stimulus which initiated digging. Such an explanation suggests a possible mechanism by which the burrowing response in bivalves is controlled.

In the Solenidae, burrowing may be initiated by stimulation of the siphons or mantle edge and may be regarded as an escape reaction, the animal retiring into its burrow in response to danger. In contrast, the escape reaction of most eulamellibranchs, including the Veneridae, is withdrawal of the siphons followed by shell closure. Burrowing occurs normally in response to disturbance; an animal removed from the substratum will, if covered with water, attempt to re-burrow. The stimulus which results in this reaction is disturbance, and possibly also exposure of the siphons and mantle edge. Animals which have been kept for some time in laboratory tanks or aquaria without a soft substratum lose this response, although they may make weak attempts to burrow at irregular intervals and often respond vigorously to a rapid change of water by extending the foot and attempting to burrow.

The cause of cessation of burrowing has not been found. Fatigue has been suggested (Fraenkel, 1927) but does not appear likely, since animals may be made to burrow repeatedly by removing them from the substratum immediately on completion of the digging-period.

The observation that the siphons maintain contact with the surface of the substratum throughout the digging-period and that extension of the siphons follows each downward movement suggests that burrowing may cease in response to stimuli originating from the siphons. Such a stimulus to be effective must give information on the state of extension of the siphons and hence involve stretch receptors or other proprioceptors. A gradual extension of the siphons during the digging-period might then result in a gradual buildup of stimuli from such receptors acting as a progressive inhibition to the burrowing response. In the Veneridae such proprioceptors, if they occur, are presumably distributed throughout the siphonal walls. In other and more active bivalves it is possible that they might form recognizable sense organs. Thus, for the Tellinacea, Yonge (1949) has suggested that the sense organ associated with the cruciform muscle may serve to give information on the state of extension of the siphons. It would be of interest to observe the digging-periods of members of this and other groups in more detail.

Thanks are extended to Dr. C. H. Mortimer for the use of facilities at The Marine Station, Millport, Isle of Cumbrae, Scotland, where some of the observations recorded here were made, and to Dr. G. Talbot, the Director; Dr. T. R. Rice, and other members of the staff of the Beaufort Laboratory of the United States Fish and Wildlife Service. The work was completed while the author was in receipt of the John Murray Travelling Studentship of the Royal Society.

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THE VITELLINE COAT OF THE MYTILUS EGG. I. NORMAL STRUCTURE AND EFFECT OF ACROSOMAL LYSIN

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The first study of a lysin extractable from *Mytilus* spermatozoa was made by Berg (1950), who distinguished two types of lytic activity: a "membrane-dissolving" action, which was described as attacking an outer layer of the egg surface made microscopically visible by plasmolyzing the eggs, and a "cement-dissolving" activity, which causes the first two blastomeres to become partially or completely separated.

Wada, Collier and Dan (1956) showed that the lysin in question is a component of the intact acrosome which is released into the medium when the acrosome is induced to react.

Colwin and Colwin (1960a, b) have also investigated the effect of a lysin extracted from the spermatozoa of *Hydroides hexagonus* on the egg of this species. Using thin sections and electron microscopy, they found that the lysin dissolves the middle, and major, component of the thick vitelline coat investing the cytoplasmic surface, although it appears not to affect the outer and inner borders of this envelope.

A report concerning the structure of the *Mytilus edulis* "egg membrane" has recently been published by Mancuso (1960). This author used a fixing solution which included formalin, acetone, acetic acid and sometimes chromic acid, as well as osmium tetroxide; the images observed in the electron microscope after this fixation led him to certain conclusions which differ considerably from those reached in this study. None of these differences, however, is so radical that it cannot be attributed to the effect of the fixative.

The present investigation was undertaken to observe the fine structure of the *Mytilus* egg surface and determine in detail how the acrosomal lysin affects it after fertilization, particularly in connection with the role of the vitelline coat in controlling the pattern of the first cleavage, and the shape and mutual relations of the first two blastomeres.

MATERIAL AND METHODS

Mytilus edulis from the Tokyo area is readily induced to spawn by keeping freshly collected animals dry in a refrigerator for several hours and then placing them in sea water at room temperature (20–23°), or by raising the temperature of the running sea water about 5° (to 18–20°) and administering an electrical stimulus, according to the method of Iwata (1949). Stimulated animals are returned to sea water in separate containers, and males which have begun to shed are stood, broad end downward, in a dry beaker to obtain concentrated sperm suspensions.

Pooled sperm from several males was used as the source of the acrosomal lysin. If 1 ml. of 0.36 M $CaCl_2$ is added to 9 ml. of rather concentrated sperm suspension, most of the spermatozoa undergo a reaction of the acrosome (see Wada *et al.*, 1956).

The sperm cells were removed by 10 minutes' centrifugation at 12,000 *g* (0°), and the clear supernatant was dialyzed against running sea water and used as the lysin. This solution retains its lytic activity indefinitely if it is kept frozen.

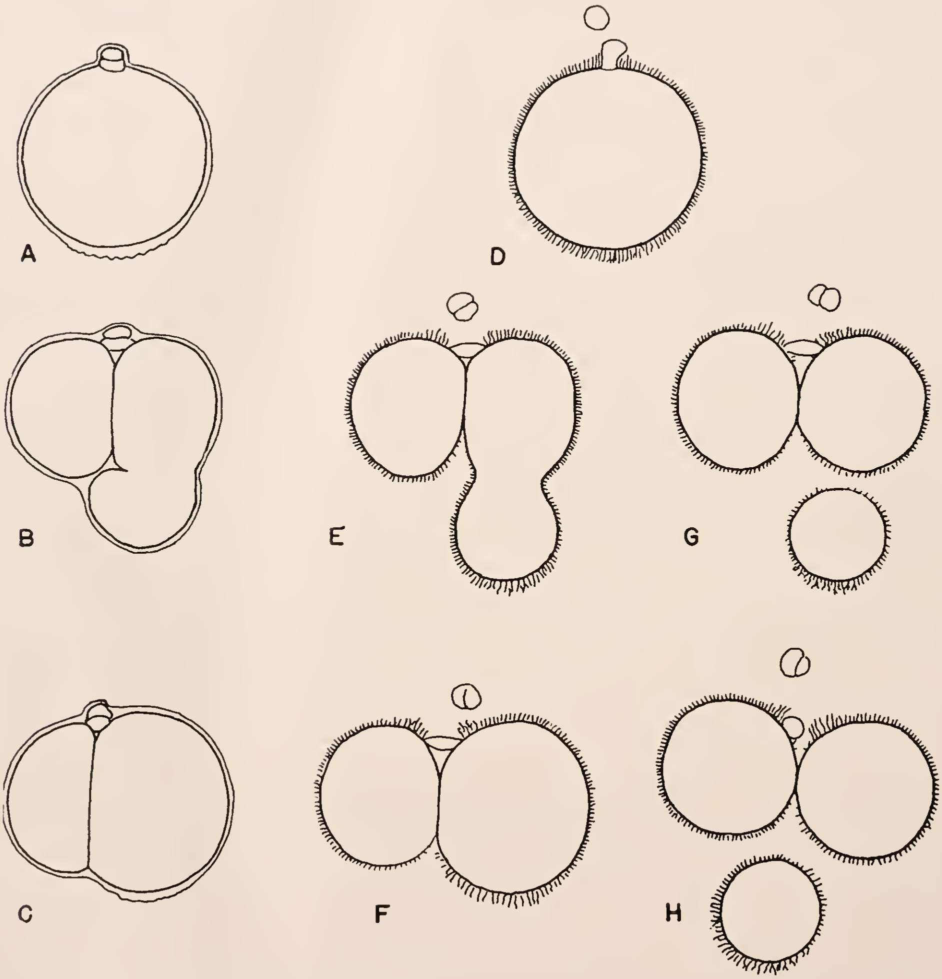


FIGURE 1. Camera lucida drawings of *Mytilus edulis* eggs cleaving under various conditions. A–C: in sea water; D–F: suspended in strong solution of acrosomal lysin; G, H: membrane removed by lysin and eggs transferred to calcium-free sea water. A, D: shortly before cleavage; B, E, G: mid-cleavage trefoil stage; C, F, H: interphase between first and second cleavages. Microvilli are visible with phase contrast as striated "halo."

All fixation was done at room temperature with 1% OsO_4 in sea water. The egg suspensions were fixed for 30 minutes, washed and post-fixed in 5% formalin-sea water for several hours, embedded in methacrylate, sectioned with a Porter-Blum microtome and observed with a JEM-5G electron microscope.

RESULTS

Living eggs

The unfertilized *Mytilus* egg is irregularly oval, and is surrounded by a conspicuous hyaline zone about 1 μ thick, which is referred to as the vitelline membrane in Field's original description (1921–1922). Outside of this "membrane" is a rather thin (7–10 μ) layer of transparent material ("jelly") which can most easily be detected by adding india ink to the egg suspension (see Wada *et al.,* 1956; Fig. 6).

On being fertilized, the egg immediately becomes spherical (diameter about 63 μ), but no change can be observed in the surface layers except that by the time of the first cleavage, the thickness of the vitelline coat appears to increase slightly.

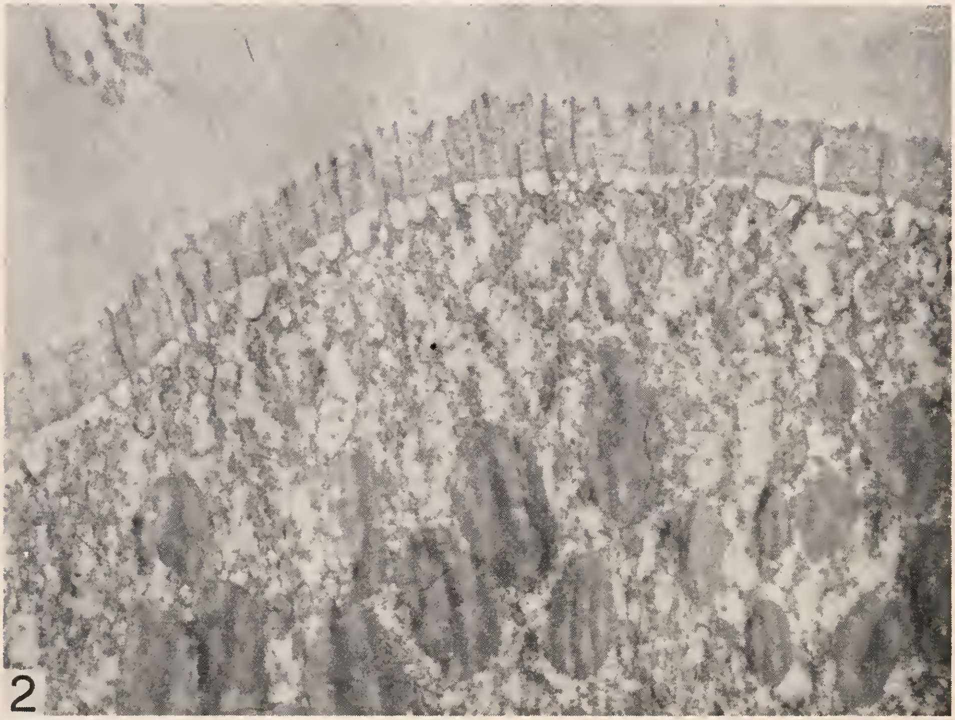
When the polar bodies are extruded, they lie under the vitelline coat, flattened against the cytoplasmic surface (Fig. 1A). At the first cleavage the egg forms a polar lobe; as cleavage proceeds, this lobe is compressed against the opposite, AB, blastomere by the tension exerted by the vitelline coat (Fig. 1B). Once cleavage is complete, the polar lobe material flows back into the CD cell, and the two blastomeres become closely apposed during the succeeding interphase (Fig. 1C).

If an egg is treated with acrosomal lysin 10 minutes after fertilization and continuously observed with phase contrast, it is seen that the vitelline coat loses first its sharp outline and then its hyaline refringency, and finally gives place to a layer of fine processes which cover the whole surface of the cell. These processes are clearly longer at the vegetal side of the egg, and also in a restricted area at the animal pole (Fig. 1D).

The first polar body bulges out freely as it is formed, and drifts away from the egg if the preparation is jarred. The second polar body remains attached to the egg surface, the first polar body usually dividing as the second is formed. At cleavage, the polar lobe extends out at right angles to the mitotic spindle (Fig. 1E); the connection between polar lobe and CD blastomere is narrower than normal, and in very strong lysin or when the eggs are transferred to calcium-free sea water after strong lysin, the connection is often severed (Fig. 1G). As Berg has reported (1950), the two resting blastomeres tend to be more spherical than those of the controls, especially after extended exposure to strong lysin, although a considerable degree of contact is more common than complete separation (Fig. 1F, H) (see also Berg, 1950; Pl. 1, c, d; Wada *et al.,* Fig. 7).

Electron microscopy

Normal egg surface. Thin sections of the unfertilized *Mytilus* egg (Fig. 2) show that its surface is similar to that of the egg of another bivalve mollusc, *Spisula,* according to an electron micrograph by Rebhun (Allen, 1958). The cytoplasmic surface is extended into fine microvilli of a relatively uniform size and regular distribution, 0.7–1 μ in length, and usually straight, although two may be connected at their bases to give an effect of branching. These microvilli extend into and through a rather dense layer, about 0.5 μ thick, of homogeneous material of the sort described by the Colwins as "felt-like," which is obviously the hyaline component of the vitelline coat as observed with light microscopy. The tips of the microvilli protrude slightly beyond the outer surface of this layer; its conspicuously smooth inner surface is separated by a definite perivitelline space from the outer border of



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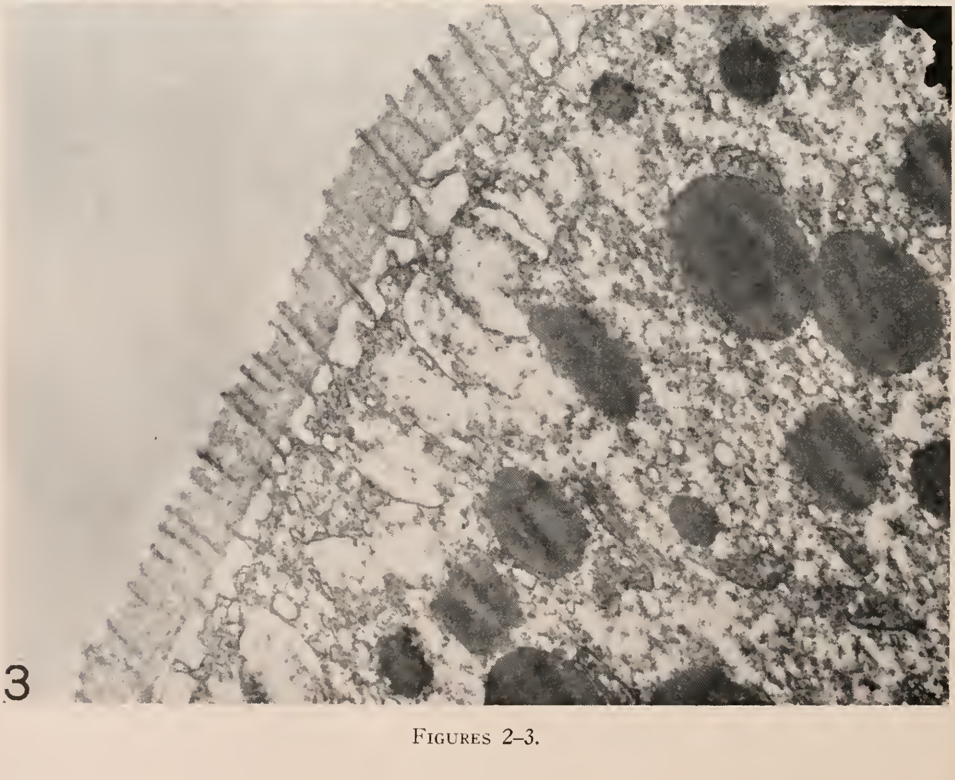

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Figures 2–3.

the cytoplasmic mass between the bases of the microvilli. From the tips of the microvilli numerous extremely fine fibrils extend outward, constituting at least one component of the so-called jelly layer.

As can be seen in Figures 2, 3 and 8, no formation which could be described as a "membrane" lies outside this layer of hyaline material, although the micrographs show regions of greater absorption, as at the right of Figure 2, which suggest that the surface of the vitelline coat is somewhat denser than its interior. A similar but more pronounced condensation is apparent in Rebhun's micrograph of the *Spisula* egg, and Mancuso's figures of the *Mytilus* egg show most of the hyaline substances concentrated into two layers, corresponding to the inner and outer surfaces of what appears in this study as a nearly homogeneous matrix.

The cytoplasm is bounded by a plasma membrane, which is continuous with the walls of the microvilli. Beneath this is a region of cortical cytoplasm 1–1.5 μ thick, generally free from yolk granules but containing conspicuous spherical membranes which in Mancuso's micrographs includes a substance having an electron absorbancy somewhat greater than that of the yolk.

Fertilization induces no changes in any of these structures that can be detected in the electron micrographs (Fig. 3). In eggs fixed between 20 and 70 minutes after fertilization, however, it is found that on parts of the egg surface, the bases of adjacent microvilli have united, while the wider spaces between these villous trunks have also deepened. The microvilli thus come to present an overall appearance of branching, but careful observation shows that their dimensions and arrangement within the vitelline coat are the same as those of the unfertilized eggs; what has changed is the intervillous cytoplasmic surface. This new state of affairs can be observed in the untreated living egg as a thickening of the vitelline coat, as mentioned above, and the greater length in the polar regions of the processes observed after lysis of the hyaline layer material is due to an extreme expression of this tendency, in all probability connected with the special role of these areas in polar lobe and polar body formation.

Lysin-treated eggs. Sections of eggs fixed after having been exposed to lysin for one minute (Fig. 4) show that the hyaline material of the vitelline coat has been evenly attacked by the lysin—*i.e.*, dissolution of the material has taken place rather uniformly throughout the layer. After an exposure of two minutes (Fig. 5), the material is virtually all dissolved, except for some vague remnants of it left clinging to the microvilli; the latter remain exposed as straight, unbranched processes, continuous with the main body of the cytoplasm and apparently unaffected by the lysin. The fine fibrils of the jelly layer are also intact (*cf.* Wada *et al.,* 1956; Fig. 7), and with the hyaline substance removed, it can be seen that there are short fibrils of the same kind projecting from the sides of the microvilli.

When eggs are exposed to the lytic solution for 10 minutes (Fig. 6), the hyaline material is completely dissolved, whereas the microvilli and the fine fibrils of the jelly are quite unaffected. The portion of the egg surface appearing in Figure 6 is

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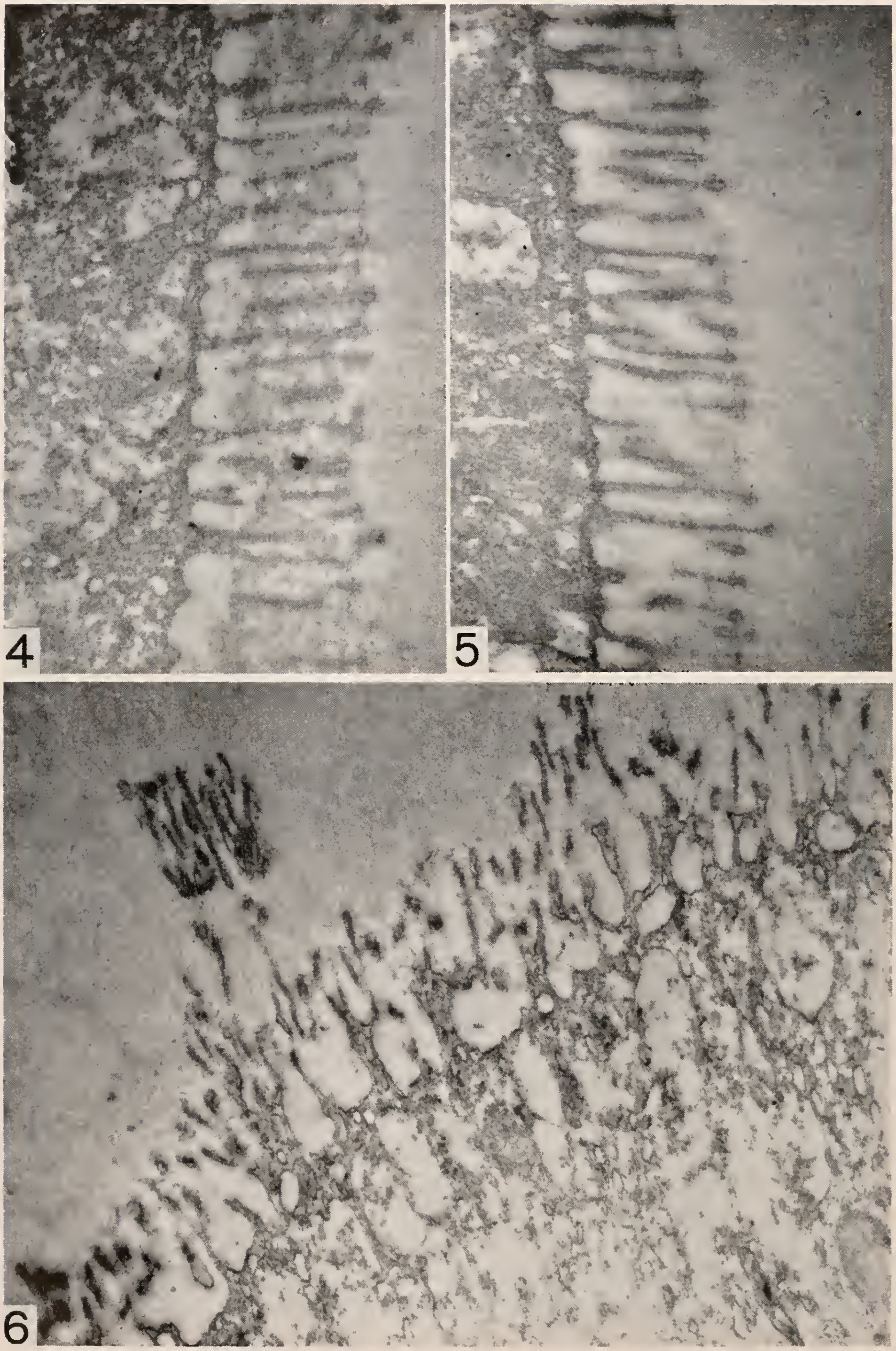
FIGURE 2. Surface of unfertilized *Mytilus* egg, showing vitelline coat consisting of hyaline material supported by microvilli. × 16,000.

FIGURE 3. Surface of fertilized *Mytilus* egg fixed 10 minutes after insemination. Note fine fibrils of "jelly layer" and empty membranes of cortical granules which have been extracted during preparation (see text). × 16,000.



Figures 4–6.

apparently from the vegetal region, since it represents an extreme case of the "branching" effect.

Further exposure to the lytic activity, up to 60 minutes, still leaves the microvilli and their fibrils unaffected (Fig. 7). The cytoplasmic protuberances carrying the microvilli in this section are coarser than those shown in Figure 6; it is not clear whether this represents a topographical characteristic or is the result of exposing the cytoplasmic surface without its supporting coat for a long period.

Lysin plus calcium-free sea water. To investigate the effect of lack of calcium on these surface structures, fertilized eggs were transferred to calcium-free artificial sea water [1] 10 minutes after insemination, as controls for another lot of fertilized eggs which were first exposed to lysin for 10 minutes and then washed with calcium-free sea water and left in it for 50 minutes (fixation at 70 minutes after insemination, shortly before beginning of first cleavage).

The calcium-free controls (Fig. 8) show no differences from the sea water controls, indicating that the integrity of the vitelline coat in these eggs is not dependent on the presence of calcium in the medium. The fibrils of the jelly layer are also found intact, both in the controls and in the sample of eggs exposed to lysin followed by calcium-free sea water (Fig. 9), and no special effect of the lack of calcium on the denuded cytoplasmic surface can be observed.

Discussion

The general structure of the *Mytilus* egg surface, as seen at high magnification, bears a surprisingly close resemblance to the surface complex of the fertilized sea urchin egg, in which secondarily-formed microvilli extend into and attach the egg surface to a hyaline layer similarly consisting of a homogeneous material (Endo, 1961). In both these systems, the overlying layer of the hyaline substance supports the cytoplasmic surface and controls the shape of the embryo as it develops through the cleavage stages.

Experiments of the sort first performed by A. R. Moore (1940), showing that sucrose freely penetrates the hyaline layer of echinoderm embryos during the cleavage stages, furnish evidence that the osmotic properties of this layer are less exclusive than those of cytoplasmic membranes. On the other hand, the fact that the width of the echinoderm hyaline layer is observed to increase just before each of the early cleavages (Dan, 1952) indicates that some osmotically active substances are retained within it. The observation presented in this study, that the *Mytilus* egg surface proper becomes indented so that the microvilli come to project from the summits of thicker cytoplasmic protuberances, suggests that a similar osmotic process is at work in these eggs, causing an increase in the volume of the perivitelline fluid. In view of the extent to which the dividing egg departs from the spherical shape, especially as it forms and retracts the polar lobes, the intervention of some such

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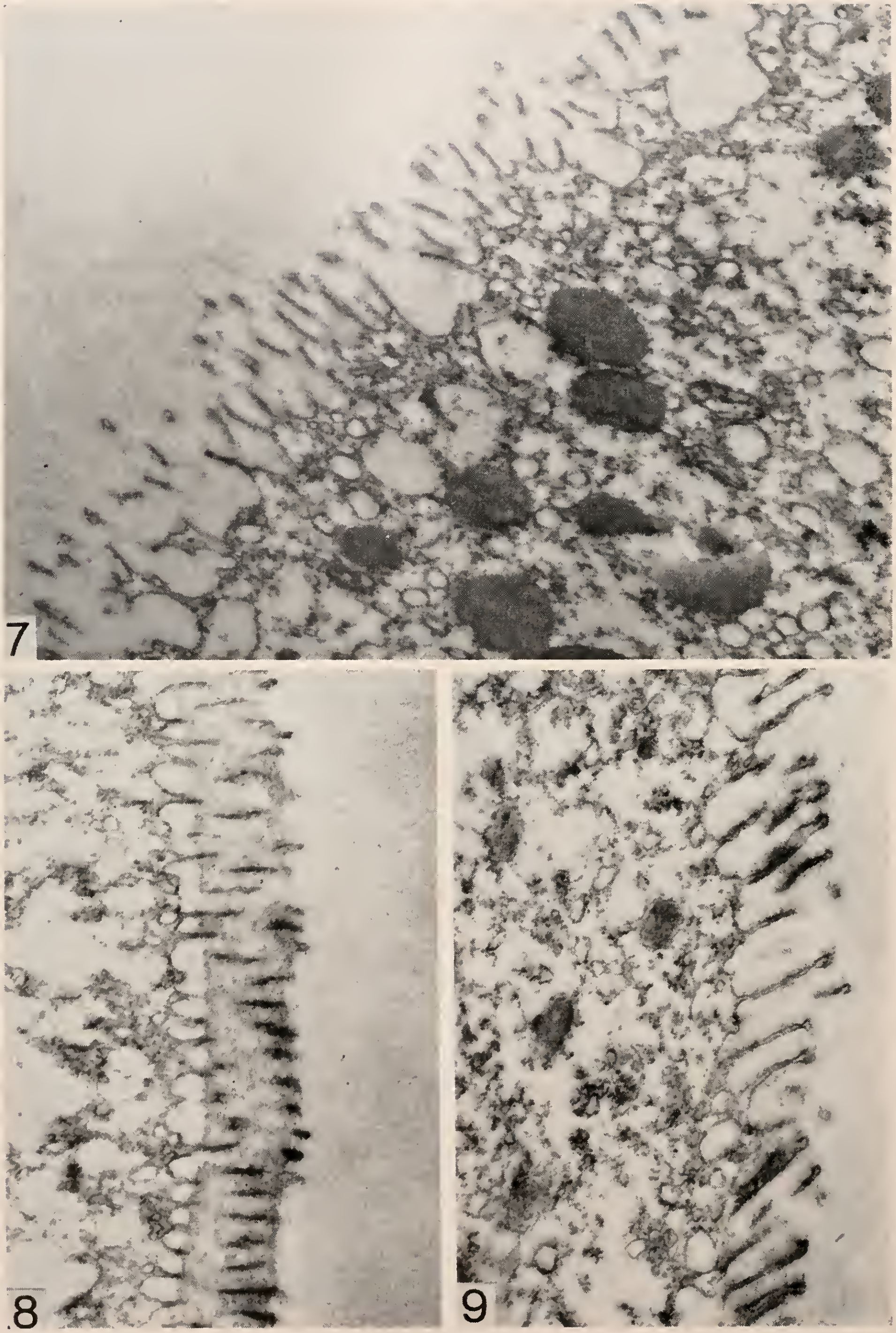
FIGURE 4. Surface of fertilized *Mytilus* egg fixed after exposure of one minute to strong acrosomal lysin. Hyaline material partly dissolved. × 24,000.

FIGURE 5. Hyaline material almost completely dissolved after two-minute exposure to lysin. Note that microvilli and fibrils of jelly layer are unaffected. × 24,000.

FIGURE 6. Vegetal surface of fertilized *Mytilus* egg after 10-minute exposure to lysin. Hyaline material completely removed; microvilli and fibrils unaffected. × 16,000.

[1] Dan's (1954) "chloride mixture No. I."



Figures 7–9.

device to reduce the restraining effect of the vitelline coat on the cytoplasmic surface would seem to be an essential prerequisite for cleavage.

That the tensile properties of the intact *Mytilus* egg surface are chiefly due to the hyaline material of its vitelline coat is suggested by the separation of the polar bodies and the considerable change in the configuration of the first cleavage following lysis of this layer (in Figure 1, compare D and E with A and B). On the other hand, the vitelline coat must be capable not only of expanding to some extent, but also of being contracted to a comparable extent, since the formation of the polar lobes involves an increase in surface area, while their retraction causes it to decrease. When the eggs are not in the best condition, wrinkling of the vitelline coat, or its complete separation from the plasma membrane as a large blister at the vegetal pole, attests to the failure of such contraction.

It is clear that the activity of the lysin derived from the sperm acrosome is specifically directed against the hyaline material of the vitelline coat, and has no effect, even after 60 minutes, on the plasma membrane. Comparing Figures 6 and 7, which both show areas of the vegetal surface, it at first appears as though prolonged exposure to the lysin has weakened the egg surface so that the slender processes supporting the clusters of microvilli in Figure 6 spread out into the thick, poorly organized protuberances seen in Figure 7. It is necessary to consider, however, that during this period the polar lobes associated with first and second polar body formation have caused the expansion of this surface in the absence of the vitelline coat, which would normally have held the distal parts of the microvilli in a fixed arrangement. It therefore seems probable, especially since the microvilli remain unchanged even after prolonged exposure, that the observed effect is secondarily produced by the absence of the supporting layer, rather than primarily, by some action of the lysin on the cytoplasmic surface.

The long microfibrils which arise from the tips of the microvilli and constitute what has been thought of as the jelly layer are interesting because of their resistance to the dissolving actions of lysin and of calcium-free sea water, and because they are fixed by osmium. The two latter characteristics set them in contrast to the mucopolysaccharide jelly of the sea urchin egg, and suggest that the zone around the *Mytilus* egg consisting of these massed fibrils should not be thought of in the same terms unless evidence can be found to indicate the presence of a more labile component.

The result of the present investigation supports the doubt which was expressed in the earlier study (Wada *et al.*, 1956) concerning Berg's (1950) suggestion that the AB and CD blastomeres are held together by a cementing substance, presumably secreted in the furrow region of the cleaving egg. It seems evident that it is rather the restraint exerted by the encircling vitelline coat which presses the blastomeres against each other in normal cleavage. That this is not the whole explanation,

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FIGURE 7. Surface of *Mytilus* egg after 60-minute exposure to strong acrosomal lysin. Microvilli and fibrils still unaffected; structure of cytoplasmic surface somewhat modified as result of prolonged absence of supporting layer of hyaline material. × 16,000.

FIGURE 8. Vitelline coat of fertilized *Mytilus* egg transferred to calcium-free sea water 10 minutes after insemination; fixed at 70 minutes. Note that hyaline material and fibrils of jelly layer are both resistant to lack of calcium. × 16,000.

FIGURE 9. Surface of fertilized *Mytilus* egg exposed for 10 minutes to strong lysin, washed in calcium-free sea water and left in this medium until just before first cleavage; fixed 70 minutes. × 16,000.

however, is shown by the observation that even when the hyaline component of this layer has been dissolved, the blastomeres preserve a considerable degree of mutual contact (Fig. 1F) unless cleavage takes place in calcium-free sea water (Fig. 1G, H).

If an analogy may be drawn between these cells and sea urchin blastomeres, which also normally have their outer surfaces attached by cytoplasmic processes to a hyaline layer (Dan and Ono, 1952), the extreme sphericity of the *Mytilus* blastomeres in calcium-free sea water after removal of their vitelline coat can be explained as an abnormal equalization of the post-cleavage membrane tension involving the whole weakened (by the absence of calcium) surfaces of the blastomeres, instead of the usual localization of such stretching in the furrow region (Dan, 1954).

Since electron microscopy shows that the outermost covering of the *Mytilus* egg is a single layer of what can be called a cementing substance or matrix material supporting and fixing in regular arrangement a brush of microvilli, rather than any structure which conforms with the usual concept of "membrane," it appears that the two lytic activities suggested by Berg (membrane-lytic and cement-lytic) would be better described as degrees of effectiveness of a single activity, combined with secondary effects of variable experimental conditions such as the degree of calcium deficiency and the length of the period during which the cytoplasmic surface is without its supporting layer.

The author acknowledges with gratitude the cooperation of Mr. A. Kitahara of the Tokyo Institute of Technology, who performed the electron microscopy.

Summary

1. Electron microscopy shows the egg of *Mytilus edulis* to be surrounded by a vitelline coat consisting of a layer about 0.5 μ thick, which corresponds to the refringent hyaline zone seen with the light microscope. This layer has a smooth inner surface, separated from the cytoplasmic surface proper by a space about 0.2 μ wide. The plasma membrane forms a brush of regularly arranged, straight microvilli 0.7–1 μ in length. These pass through and protrude slightly beyond the outer surface of the hyaline material, where their tips give rise to numerous extremely delicate fibrils which constitute at least one component of the "jelly layer." Fertilization does not cause any visible changes in these structures of the egg surface.

2. Exposure of a fertilized egg for one minute to a strong solution of acrosomal lysin causes an evident dissolution of the hyaline substance, and a two-minute exposure removes it almost completely, leaving the microvilli exposed but otherwise unaffected. The fibrils of the jelly layer also resist the lytic action. Exposure to lysin for 60 minutes induces no further changes in these structures.

3. It is concluded that the acrosomal lysin is specific for the single substance constituting the hyaline portion of the vitelline coat, and that the layer composed of this material is chiefly responsible for the configuration of the cleaving egg and the close contact of the blastomeres after cleavage.

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MIGRATORY RESTLESSNESS IN CAGED BOBOLINKS (DOLICHONYX ORYZIVORUS, A TRANSEQUATORIAL MIGRANT)[1]

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It has been known for a very long time that caged migratory birds become increasingly restless at the onset of the migratory season. This restlessness is expressed especially by fluttering and hopping on the perches after dark when, ordinarily, caged birds sit quietly. Most investigators have assumed, at least tacitly, that the development of such nocturnal restlessness reflects the development of a disposition to migrate. It is frequently referred to as *Zugunruhe* (migratory unrest). Thus equated with migratory behavior, it could be a useful indicator in various aspects of the experimental study of migration (*e.g.,* regulation, physiology, navigation).

In the past three decades there have been a number of studies on the induction and regulation of this restlessness with respect to such factors as temperature, food, sex hormones, and photoperiod. Although one may doubt in some cases that the restlessness truly reflected a migratory state, there is clear evidence for the photoperiodic induction of migratory behavior in at least a few temperate zone migrants (*e.g.,* King, 1961, *Zonotrichia leucophrys gambelii*). Transequatorial migrants have received little attention although obviously they are of special interest in relation to photoperiodism. Each of their two annual migrations, northward and southward, begins during the declining day-lengths of late summer or early autumn in one hemisphere and ends during the increasingly longer days of middle or late spring in the other. In his studies on star-navigation by birds Sauer (1957) has employed restlessness in the European-African transequatorial migrant *Sylvia borin* to determine directional orientation. Recently Hamilton (1962) demonstrated orientation under clear skies in the nocturnal restlessness of caged bobolinks during both spring and fall migratory periods. Neither of these was concerned with the induction or regulation of the restlessness *per se,* although an intrinsic rhythm seems to be implied for *S. borin* (Sauer and Sauer, 1955).

It has already been shown that the annual testicular cycle of caged bobolinks (*Dolichonyx oryzivorus*) is under photoperiodic control (Engels, 1959, 1961). This species breeds in North America above Lat. 40° N., reaching the nesting area in late May or very early June after a migration, begun in late March or early April, from a "wintering" area in South America lying roughly between Lat. 10° and 30° S. It is then of some interest to determine if caged bobolinks display a seasonal nocturnal restlessness which may be related to migratory behavior and controlled or influenced in any way by day-length.

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[1] This study was supported by a grant (G-6163) from the National Science Foundation.

Materials and Methods

The bobolinks used in these experiments were captured in North Carolina at about Lat. 36° N., some in May, others in September, thus near the end of the northward or shortly after the beginning of the southward migration. These are referred to hereafter as "spring captures" and "autumn captures," respectively. Some individuals were used as experimental animals in successive years; after the first year these are designated "2nd year experimentals." Each such bird had spent at least six months (June through November) in an outdoor aviary exposed to natural day-lengths before being used in another experiment.

Over a three-year period four groups of birds were used, confined in outdoor aviaries exposed to normal outside air temperatures. Of these, two groups experienced only natural day-lengths (Lat. 36° N.), while two were exposed to constant 14-hour photoperiods beginning in November. The birds were confined individually in small cages placed on a shelf in the sheltered rear part of the otherwise open aviaries.

The cages used were "Hendryx finch-breeder" cages measuring about 8 × 9 × 16 inches. Each was fitted with two perches which pivoted at one end on a horizontal metal rod ($\frac{1}{8}$ inch diameter) placed about three inches outside the rear of the cage and attached to it at each end by a frame. At the front each perch rested on a microswitch attached to the cage. Closure of either switch, resulting from a depression of the perch as the bird hopped on it, actuated an electronic counting device,[2] each closure advancing a four-digit counter by one. Every 15 minutes the accumulated count automatically was printed on a clock-motor-driven, chronologically marked tape, and the counter then returned to zero.

To each cage were attached a food-hopper and two 100-cc. water tubes which provided at one filling sufficient food and water to supply a bobolink of normal behavior for more than a week. A small amount of soluble terramycin was added to the drinking water. At first a chick laying-mash (Purina Layena) was used as food; we later changed to a mash prepared for game birds (also Purina). The pan of the cage was covered with a layer of finely crushed granite.

The birds were left undisturbed except for periodic handling for weighing and inspection of the plumage. This was done usually once a week. On these occasions the cages and water tubes were cleaned, fresh water and clean granite supplied and the food-hopper refilled. Body weight was determined on a balance reading to the nearest 0.01 gram and recorded to the nearest 0.1 gram.

An enormous amount of raw data on perch hopping activity was accumulated over the three seasons of observation. To reduce this to manageable and meaningful figures, three indices were used, as follows:

Index A: This is simply the number of quarter-hour periods during the night in which one or more perch movements were recorded. (This is similar to the index used by Weise, 1956, but his interval was a more satisfactory 0.1 hour). Such periods may be designated as *active night periods*. Selection of the quarter-hour as the basic interval was dictated by the recording device, which automatically printed out the number of perch movements every 15 minutes. *Night* here means the total dark period for birds exposed to artificial illumination, or, for birds exposed to natural photoperiods, the interval between the end of evening civil twilight and the beginning of morning civil twilight. Obviously, one hop per quarter-hour is hardly indicative of restlessness, but the index has usefulness and value when combined with the next index (B), as giving some information on the possible maximum duration of unrest during the night.

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[2] "Tally-Print," Model AR, Standard Instrument Corp., New York.

Index B: This is the average number of perch movements (hops) recorded per active night period and thus is an expansion of Index A.

Index C: This is the total number of perch movements (hops) recorded during the night, the product of the first two indices.

I would like to acknowledge, with thanks, the technical assistance of Donald E. Kent, especially in the design and construction of the perch-microswitch arrangements and in maintenance of the electronic recorders, and the help of Catherine Henley in the preparation of the manuscript. The figures were prepared by Mary Scroggs.

RESULTS

Experiment 1 (Table I)

During the fall, winter and spring of 1959–60, essentially continuous records of perch-hopping activity were obtained for four males confined in an outdoor aviary and thus exposed to essentially natural day-lengths and normal air temperatures.

TABLE I

Summary of nocturnal activity of four male bobolinks in relation to season, natural photoperiod, and ambient temperature, as measured by recorded perch-hopping movements, January 3 to May 3, 1960, Chapel Hill, N. C., Lat. 36° N. Outdoor aviary, natural lighting only

Week ending	Air T ° C.		Nocturnal Activity Indices		
	Aver. H	Aver. L	A	B	C
Jan. 10	9	−1	1.4	3.0	4
17	18	+4	9.9	21.2	210
24	6	−6	2.2	7.6	17
31	11	−1	6.0	5.9	35
Feb. 7	11	−1	6.5	13.1	85
14	13	−1	1.9	3.8	7
21	8	−4	<1	1.5	<1
28	9	−3	1.2	5.0	6
Mar. 6	4	−6	<1	5.0	<1
13	2	−8	<1	2.5	<1
20	7	−2	3.0	19.4	58
27	12	−3	17.6	41.4	729
Apr. 3	23	+9	17.3	79.0	1367
9	21	7	25.7	54.2	1393
19	23	8		*no records*	
26	28	12	22.8	66.6	1518
May 3	23	8	24.9	86.1	2144

Nocturnal Activity Index A: average number of active quarter-hour periods per bird per night (one or more hops recorded during the quarter-hour period); B: average number of hops recorded per active quarter-hour night period; C: average number of hops per bird per night. See text.

The photoperiod (including twilight) increased from a low of about 10½ hours in December to about 15 hours in May. Two of the four males were "autumn captures," one was a "spring capture" and one a "2nd year experimental." The latter two both had experienced natural day-lengths through the summer of 1959. (These are the birds designated as "Group F" in Engels, 1961, Table II, p. 143.)

Through the autumn these birds displayed bursts of nocturnal activity in a somewhat sporadic manner, but this eventually declined in intensity, more or less coincident with the advent of cold weather. At this time diurnal activity also declined, so that frequently an individual bird did not record more than 100 perch movements in a 24-hour period. The nocturnal activity indices for these birds, from January 4 to May 4, are presented as weekly averages in Table I, together with average high and low air temperatures as recorded by the local station of the U. S. Weather Bureau. It will be noticed that all indices have low values from January on through the week ending March 20. The slight elevation of the indices for the week ending January 17 might be a reflection of the slight rise, about 6° C., in average ambient temperature. However, the sharp rise in all indices apparent for the week ending March 27 occurred while the average low (night) air temperatures were still below the freezing point. Although the continued maintenance of intensive perch-hopping activity during the nights of succeeding weeks coincided with the normal spring rise in air temperature, the whole picture does suggest a nocturnal restlessness induced by something other than an increase in environmental temperature.

The intensification of nocturnal activity was also more or less coincident with a photoperiodically induced production of male sex hormone. This latter event is manifested in bobolinks by a change in the pigmentation of the horny beak, which eventually becomes a deep glossy black (Engels, 1959, p. 761). With one exception the onset of nocturnal restlessness was abrupt, occurring in a single night. The actual dates for the individual birds were the nights of March 19/20, 23/24 and April 5/6. For one bird restlessness began also on March 23/24, then slacked off after a few nights but was persistent every night after April 3/4. In two cases this onset of restlessness preceded the initial appearance of beak pigmentation by at least a week, in one case by at least three days, and in the fourth bird the two events may have been essentially coincident.

Two of the four birds went through an almost complete prenuptial molt. In both, molting was intense and general in early February and continued on into mid-March. During this period body weight diminished by about 10 grams or by about 20 to 25%. In both, nocturnal restlessness appeared within one or two weeks of cessation of molt and shortly following an upturn in body weight. In the other two birds the prenuptial molt was very incomplete, a few new replace-feathers appearing in middle to late March and early April. However, both lost weight during this time, approximately to the same extent as the birds described above. In both, the beginning of nocturnal restlessness occurred more or less coincidentally with an upturn in body weight.

In view of the varied previous history of these four birds, it should be mentioned that of the two "autumn captures" one went through an essentially complete molt resulting in typical cock plumage, the other remained essentially henny in appearance. Restlessness developed earliest (March 19/20) in the male caught in the previous spring; it set in only three days later (March 23/24) in the "2nd year experimental" and in one of the two "autumn captures."

During this same winter, activity records were being obtained for a number of bobolinks caged indoors where night air temperatures never fell below about 11° C. Some of these birds were persistently active at night during December, when the outdoor birds had practically given up all exercise. In January an exchange was made, transferring an exceptionally active female to the outdoor aviary in place of one of the males, and putting him into her cage in the laboratory attic. The change in photoperiod was slight, from a constant 10 hours light-14 hours dark in the attic to approximately 11 hours light-13 hours dark in the aviary. But there was a complete, almost dramatic reversal in the nocturnal activity performances of the two birds as they were shifted back and forth between the warmer and colder environments (Table II). Looking at these data, it is impossible not to suspect that temperature here is playing a decisive role in regulating the extent and degree of nocturnal activity.

TABLE II

Apparent effect of ambient temperatures on the nocturnal activity of two bobolinks, in an outdoor aviary (natural photoperiods, Lat. 36° N., outdoor temperatures) and in the laboratory attic (constant 10-hour photoperiod, 14 hours darkness daily, night temperatures from about 11° C. to about 17° C.). Activity Indices as in Table I.

Periods	Outside temp. ° C.		Nocturnal Activity Indices					
	Aver. H	Aver. L	♂81			♀68		
			A	B	C	A	B	C
				in aviary			in attic	
Dec. 27–Jan. 7	14	−1	0	0	0	34.7	59.0	2047
Jan. 3–8	8	−1	2	<1	2	26.3	54.1	1423
				in attic			in aviary	
9–15	15	+3	0	0	0	6.9	5.0	35
16–23	12	0	32.1	71.2	2286	3.0	4.8	14
24–29	7	−6	27.9	89.2	2489	2.8	5.0	14
Jan. 30–Feb. 3	9	+2	4.6	56.3	259	5.0	5.0	25
Feb. 4–10	11	−2	42.3	141.2	5973	3.9	3.0	12
				in aviary			in attic	
11–17	11	−1	3.4	6.2	21	24.6	60.9	1498
18–24	4	−3	0	0	0	30.0	64.6	1938

In view of the subsequent development of nocturnal restlessness in the aviary birds (Table I) while night air temperatures were still regularly dropping below the freezing point, it seemed logical to test the hypothesis that this activity had been photoperiodically induced by exposing some birds at low air temperatures to long, others to short photoperiods. If warmer temperatures promote nocturnal activity in these birds when caged, and colder temperatures inhibit or suppress it, could a photoperiodic response "break through" and express itself as persistent perch-hopping during the night despite low air temperature? In the absence of suitable low-temperature control facilities, it was decided to make use of normal winter temperatures and to repeat the outdoor aviary experiments, suitably modified, in the following year.

Experiment 2 (Table III, Figures 1 and 2)

In 1960–61 two aviaries were used and records of activity obtained for six males, three in each aviary. The aviaries were illuminated only by natural light until November 27. After this date one aviary continued to receive only natural light, while in the other white fluorescent lights (minimum intensity about 35 foot-candles at perch level) burned daily from 5:15 AM to 7:15 PM. The constant artificial photoperiod was thus 14 hours, which previously had been shown to be stimulatory in the testicular photoperiodic response of bobolinks when preceded by several weeks of shorter photoperiods (Engels, 1961). In the naturally lighted aviary the photoperiods declined to about 10½ hours at the December solstice, then gradually increased but did not reach 14 hours until mid-April.

Table III

Summary of nocturnal activity of two groups of bobolinks (3 ♂♂ each) in relation to season, natural and lengthened photoperiods, and ambient temperature, as measured by recorded perch-hopping movements January 3 to May 2, 1961, Chapel Hill, N. C., Lat. 36° N.

Week ending	Air T ° C.		Nocturnal Activity Indices					
	Aver. H	Aver. L	Group AvN			Group AvL		
			A	B	C	A	B	C
Jan. 10	11	−5	2.9	9.1	26	1.1	1.6	2
17	12	−2	2.1	3.1	7	1.3	11.2	15
24	7	−6	2.7	2.7	7	1.0	1.3	1
31	3	−9	1.4	1.8	3	<1	2.0	<1
Feb. 7	6	−7	3.5	11.9	42	1.0	3.7	4
14	11	−2	4.6	5.5	25	1.2	8.8	11
21	19	+4	6.6	9.6	63	8.6	25.9	223
28	18	3	2.8	10.2	29	11.5	19.6	225
Mar. 7	20	7	7.7	20.7	159	19.0	35.4	673
14	19	5	8.4	19.7	165	16.5	40.9	675
21	15	1	10.6	18.3	194	18.8	41.0	771
28	14	1	25.3	27.9	706	26.1	50.1	1308
Apr. 4	18	4	29.3	37.4	1096	25.0	46.5	1163
11	16	2	27.8	38.1	1059	24.3	37.1	1273
18	18	4	17.6	30.1	530	22.9	38.5	882
25	21	8	31.0	45.9	1423	24.5	43.4	1066
May 2	22	9	31.1	38.3	1191	16.5*	35.2*	581*

Group AvN, outdoor aviary, natural illumination only; Group AvL, outdoor aviary, extra, artificial lighting, in addition to natural light, 5:15 A.M. to 7:15 P.M. daily from November 28 to April 20. Activity Indices as in Table I.

* One bird apparently becoming inactive at night this week; no later records.

None of the bobolinks used was newly captured. However, two of the three birds in each group were in their second year in the aviary under continuously natural day-lengths, and of these, one in each group had never been exposed to artificial photoperiods.

The results are given in Table III. The winter was much more mild than the previous one, the weekly average low air temperatures remaining above freezing after mid-February, whereas in 1960 they remained below freezing throughout March. Nevertheless, a marked rise in the nocturnal activity indices for the naturally lighted birds (Group AvN) occurred in the same week, the last week in March, as it had for the aviary birds in the preceding year (*cf.* Table I). On the other hand, in the aviary birds exposed to 14-hour photoperiods this pronounced increase in nocturnal activity occurred about three weeks earlier.

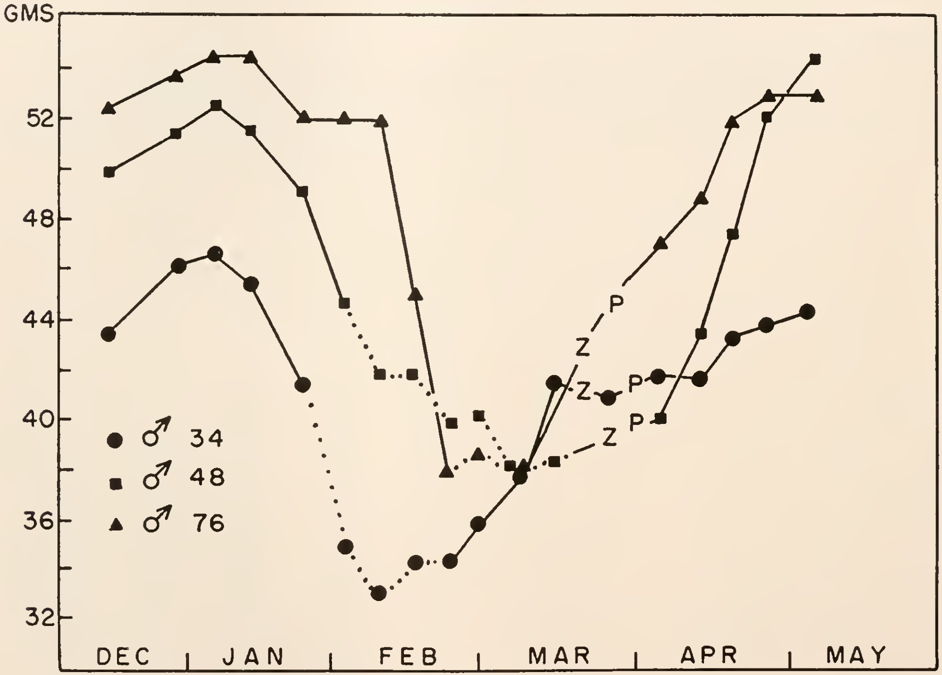

FIGURE 1. Variation in body weight of 3 male bobolinks in an outdoor aviary, Lat. 36° N., natural photoperiods; group AvN of Table III. Dotted lines indicate periods of molt; P: approximate time of beginning of nuptial pigmentation in the beak indicative of testicular recrudescence; Z: approximate time of onset of pronounced nocturnal restlessness. Body weights determined (usually) at one-week intervals.

This appears to be a definite acceleration of the cycle, which may be attributed to the lengthened photoperiod. Considering the long period of exposure to long days, about three months, before the response occurred, the acceleration seems slight indeed. Yet this result might have been anticipated from our previous studies on the testicular cycle of bobolinks, which demonstrated a long delay in the testicular response to photoperiodic stimulation (Engels, 1961, pp. 144–145).

The lean weight of male bobolinks is 30 grams or less. Birds weighing 40 grams or more are conspicuously fat, especially over the rump and in the abdominal region. As shown in Figures 1 and 2, all of the birds were very heavy through December and early January. All but one experienced sharp losses in body weight between the end of January and the middle of February. Each of these also went through an almost complete prenuptial molt to the cock plumage. This molt did not occur in the single bird in which body weight remained high (no. 30, Fig. 2). The five birds which had lost weight, and which had molted, developed nocturnal restlessness subsequent to the molt and either just prior to or shortly after the beginning of increase in body weight. In all cases but one, the onset of nocturnal restlessness preceded the development of beak pigmentation, as in the four aviary birds of the previous year, described above. One male (no. 28, Fig. 2) did not develop nocturnal restlessness until about 10 days after the beginning of peak pigmentation.

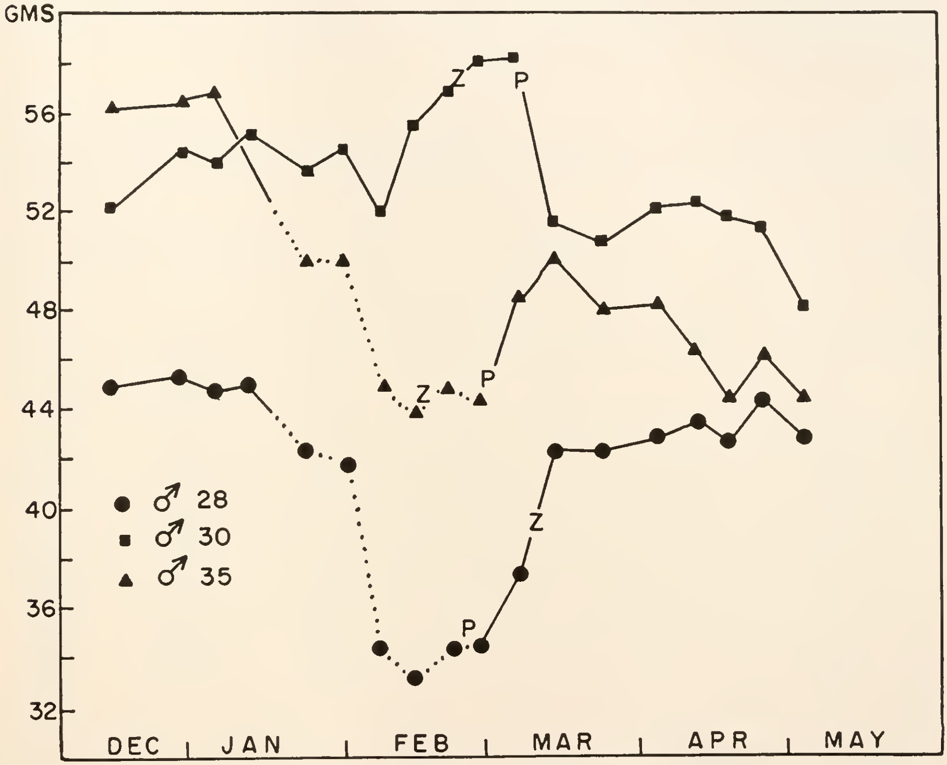

FIGURE 2. Variation in body weight of 3 male bobolinks in an outdoor aviary, as in Figure 1, but exposed to 14-hour photoperiods (5:15 A.M. to 7:15 P.M.) daily, beginning November 28; group AvL of Table III. (See also legend of Figure 1.)

Experiment 3 (Table IV)

This was essentially a duplication of part of the second experiment, using only "spring captures." Three males captured in the spring of 1961 were confined in an outdoor aviary. Beginning November 15, 1961, a 14-hour photoperiod (5:15 AM.–7:15 PM) was superimposed on the natural day-length (white fluorescent lights; minimum intensity at perch level about 35 f.c.). A single perch in each cage was balanced on the tip of the 6-inch actuating arm of a microswitch. Perch-hopping activity was relatively infrequent at night until after the middle of March when restlessness became pronounced. There again appeared to be some correlation with ambient temperature, with flurries of restlessness, appearing during the weeks ending January 28 and February 25, associated with rises of air temperature. Throughout the period of recording, it was only when the weekly average of the daily mean air temperature exceeded 9° C. that the average index A was above 16 (= 4 hours), index B above 15 (= 1 hop per minute), and index C above 400 (hops per bird per night).

Table IV

Nocturnal activity of 3 male bobolinks in an outdoor aviary at Chapel Hill, N. C., Jan. 7-Apr. 22, 1962; 14-hour photoperiod (5:15 AM–7:15 PM) superimposed on natural day-lengths beginning Nov. 15, 1961

Week ending	Air T ° C. average			Nocturnal Activity Indices		
	H	*m*	L	A	B	C
Jan. 14	3	−3	−9	3.2	10.0	32
21	7	1	−5	6.3	7.4	47
28	16	10	+3	9.8	22.6	221
Feb. 4	9	3	−4	2.1	9.0	19
11	10	3	−3	4.5	6.3	28
18	9	3	−3	2.7	11.7	30
25	16	10	+4	6.9	39.7	274
Mar. 4	11	6	+1	8.4	11.5	97
11	6	2	−1	1.8	3.7	7
18	13	7	+1	12.5	10.8	135
25	17	10	3	22.6	19.1	432
Apr. 1	20	13	6	19.0	27.3	519
8	18	12	5	20.2	27.0	545
15	19	14	8	20.7	18.3	379
22	24	15	5	18.3	24.2	443

One bird developed the black pigmentation of the beak characteristic of testicular recrudescence during the week ending February 28. The same bird abruptly became restless at night about two weeks later on March 11/12. The other two males both developed black pigment in the beak during the week ending March 8 and a very distinct beginning of heightened restlessness on the night of March 18/19.

Discussion

The primary question is: "Does pronounced nocturnal restlessness in these caged bobolinks reflect a disposition to migrate?" Questions as to the induction and regulation of the restlessness must remain secondary, and academic, until the first is answered. The results of the present experiments are not unequivocal.

The relatively abrupt change in nocturnal behavior in all experiments occurred during March. Northward migration of free-living birds probably begins in the latter part of March and early April. (The first flocks of migrating males usually arrive in the coastal areas of the southernmost U. S. shortly after the middle of April. I have seen three male specimens taken between Lat. 22° S. and 17° S., within the "wintering" area, on March 23, 28 and April 1.) Had the experiments taken place in South America, the near coincidence of the onset of restlessness with the migratory season might be interpreted as meaningful.

There was also some degree of correlation between the onset of pronounced nocturnal restlessness and some other cyclic events which are associated with migration, namely molt, fattening, and testicular recrudescence. *None* of the several museum specimens I have seen from the "wintering" grounds has the black beak indicative of testicular recrudescence, even though three were collected (between Lat. 22° S. and 17° S.) as late as the last week of March. Moreover, thirteen South American April specimens definitely were migrants, and of these, nine (northern Brazil, Venezuela) likewise had light-colored beaks. In the other four (Colombia) the beak was darkening at the time (collector's hand-written notes on labels indicated color of "mandible" as "gray," "light gray," and [two] "gray with border and point black," respectively).[3] Evidently migration gets underway before the blackening of the beak. In our experiments, pronounced nocturnal restlessness set in after the change in beak pigmentation in only four of thirteen cases, in another one the two events occurred more or less simultaneously, while in eight the onset of restlessness occurred before the change in beak pigmentation, just as in nature the beginning of migration precedes this event.

A molt from the winter "henny" plumage to the nuptial "cock" plumage may be complete by the end of January (University of Michigan #90875) but apparently more usually occurs during February and March. At any rate it is completed before northward migration begins. Presumably premigratory fattening occurs subsequent to the molt, but I have no information about this. (Datum on weight is given on the label of only one of almost 100 museum specimens, known to me, taken south of the U. S. Spring migrants taken in the southern U. S. are conspicuously fat.)

Our caged birds tended to remain fat throughout the year, except during periods of molt. Molt in these birds presents a puzzling problem. In some the molt to nuptial or "cock" plumage was essentially complete, in some it was partial, in some it was more or less completely suppressed. These differences appeared among birds with identical previous history of capture and treatment. However, when molt was complete (or partial), it occurred prior both to the onset of pronounced nocturnal restlessness and to the development of black beak pigmentation. Following the molt there was always a sharp rise in body weight, caused by the deposition of fat. The onset of pronounced nocturnal restlessness was always associated with this rise in body weight.

All of these observations, associating nocturnal restlessness with season, fattening, molt, and testicular recrudescence in a general temporal sequence comparable to that obtaining in nature, at least suggest that the restlessness of the caged birds reflects a true migratory unrest. Since there is no good reason to suppose that these various events are all causally related, the few discrepancies in sequence in the experimental birds are not unexpected. The mechanisms responsible for each may proceed from different thresholds, from different stimuli, at different times and along different pathways, the natural coordination of which may be upset by the experimental treatment. One may surmise, for example, the existence of an antagonism such that if the hypothalamic-hypophyseal gonadotropic mechanism gets started a bit relatively early in a particular captive individual, the mechanism(s) leading to molt may be partially or fully inhibited.

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[3] Grateful acknowledgment is here made to the following for the loan of specimens: Dean Amadon (American Museum of Natural History); Kenneth C. Parkes (Carnegie Museum); Emmet R. Blake (Chicago Natural History Museum); Harrison B. Tordoff (Museum of Zoology, University of Michigan); James Bond (Philadelphia Academy of Sciences); P. S. Humphrey and Mary A. Heimerdinger (Peabody Museum of Natural History).

In studies on temperate-zone migrants, the experimental birds are obtained on the wintering grounds, after the fall migration has been completed. Our bobolinks were caught in May and in September, and hence their southward migration was prohibited. That a nocturnal restlessness tended to persist in them is not surprising since it is a common observation that caged birds under natural photoperiods remain in a state of migratory unrest far beyond the natural migratory period, often until the next molt (*cf.* Farner, 1960). The restlessness in our bobolinks caged out of doors was inhibited seemingly by low environmental temperatures (*cf.* Table II). Nevertheless (in Experiment 1, Table I) pronounced restlessness set in, during March, when air temperatures were still below freezing every night and the daily mean temperature averaged only about 5° C. As Schildmacher (1938) concluded from rather comparable observations on European robins (*Erithacus rubecula*), ". . . one must assume that in these birds the effect of [an inductive mechanism] overweighed the effect of low temperature" (p. 151, free translation).

In the quoted statement Schildmacher actually specified "lengthened daylight" as the inductor. That lengthened daylight may play a role in the development of nocturnal unrest in bobolinks is indicated by the results of our Experiment 2 which show that this activity appeared earlier in birds exposed to 14-hour photoperiods as compared to those experiencing only natural (winter) photoperiods (Table III). Farner (1960) has reported positive results in similar experiments with *Zonotrichia leucophrys gambelii;* a chief difference in the data lies in the lapse of time between the first 14-hour photoperiod and the Zugunruhe response—a few weeks for *Z. l. gambelii* (a temperate-zone migrant), more than three months for bobolinks. A similar delay appears in the photoperiodic induction of testicular development in bobolinks (Engels, 1961; also Fig. 2, above); it seems to be an essential part of this species' adaptation to the long days it experiences in the southern hemisphere between breeding seasons.

A. J. Marshall, a notable and vigorous protagonist of the idea that photoperiod is concerned with reproduction of birds only insofar as it may influence an internal rhythm, has stated (Marshall, 1961, p. 331) that "Spring *Zugunruhe* is a behavior pattern that is undeniably activated by photostimulation and probably by testosterone." If genuine migratory unrest in bobolinks is in any way activated by testosterone, either the testosterone must be stimulatory at a threshold concentration far below that which produces a change in pigmentation of the beak in the male or the behavior response must occur much more quickly, since, on the evidence of museum specimens, bobolinks in their northward migration may reach northern South America without showing this change. Doubt is cast on any testosterone activation by those experimental cases in which the beak became black as much as two weeks before the onset of pronounced nocturnal restlessness (Experiment 3).

It should be admitted that, from the evidence obtained thus far, the possibility is not precluded that annual cycles in this transequatorial migrant may rest fundamentally on an internal rhythm as "the primary seasonal initiator" (Marshall, 1961, p. 309). Actually, the very long delay in response to 14-hour photoperiods, the slight acceleration as compared to natural (winter) photoperiods (Experiment 2), might readily be interpreted in terms of this concept.

SUMMARY

1. The bobolink (*Dolichonyx oryzivorus*) is a transequatorial migrant which breeds (June-July) above Lat. 40° N. in North America and "winters" (November through March) below about Lat. 10° S. in South America. The northward migration occurs during April and May.

2. Observations on nocturnal restlessness, molt, body weight and the testicular cycle were made on some captive bobolinks at Lat. 36° N., caged out-of-doors and exposed to natural as well as to lengthened photoperiods, and to normal outdoor air temperatures. "Restlessness" was recorded every quarter-hour, by an electronic counting device, as the number of hops made by each bird on the perches of its cage.

3. Restlessness was almost completely suppressed by the low air temperatures of winter. Nevertheless, intense nocturnal unrest set in rather abruptly in late March when air temperatures at night were still regularly below the freezing point (natural photoperiods).

4. When 14-hour photoperiods were superimposed on natural day-lengths, beginning November 28, restlessness set in about three weeks earlier than in the controls which experienced only natural day-lengths. This was interpreted as evidence at least of a photoperiodic influence on, if not photoperiodic induction of, nocturnal restlessness.

5. The onset of pronounced unrest was always associated with a marked rise in body weight, due to the deposition of subcutaneous and intraperitoneal fat, usually following a molt. In most cases this onset of restlessness preceded, by an appreciable interval, the appearance of black beak pigmentation, which indicates testicular recrudescence. These temporal relationships correspond to a sequence of events in nature and are tentatively interpreted to mean that the restlessness reflects the induction of a migratory state.

6. The long delay (about three months) in the response to the presumed stimulation by long photoperiods is similar to the delay found in the response of the testicular cycle to photostimulation. An interpretation in terms of an internal rhythm as a primary seasonal initiator, with other factors (such as photoperiod), acting as accelerators-inhibitors, is not precluded.

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ANAEROBIC GLYCOLYSIS IN AMPHIBIAN DEVELOPMENT[1]

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Under anaerobic conditions, the embryos of frogs probably degrade carbohydrate to lactic acid by a sequence of chemical reactions of the Embden-Meyerhof type. The operation of the sequence (glycolysis) presumably is able to generate some or all of the energy required for cleavage under anaerobiosis (Brachet, 1934). It may even meet the energetic demands of anaerobic gastrulation (Gregg and Kahlbrock, 1957), although Brachet (1960) has pointed out that the evidence is conflicting. In any case, it would appear to be an important device for sustaining embryos in straitened respiratory circumstances, of frequent occurrence in the interior of large clumps of naturally oviposited eggs. It is surprising, therefore, to find that little attention has been given to systematic study of the glycolytic capacities of frog embryos. A few papers on the subject exist in the literature. But some of these (Lennerstrand, 1933; Brachet, 1934) were published before the exigencies of rearing embryos under strictly *aerobic* conditions were understood or overcome, and are subject to still other criticisms noted by Cohen (1954). Others, including Cohen's paper and the earlier one of Barth (1946), cover only rather narrowly circumscribed morphogenetic periods. We are thus without a complete picture of the glycolytic behavior of pre-hatching frog embryos.

This paper has two purposes. The first is to take some steps toward filling the hiatus mentioned above, by surveying the glycolytic activity of developing *Rana pipiens* embryos. The second is to explore more thoroughly than before the reduced glycolytic activity exhibited by gastrula-arrested hybrid embryos obtained by fertilizing *Rana pipiens* eggs with *Rana sylvatica* sperm. Hybrids of this type were first studied by Moore (1946), from a morphological point of view. Later analysis has provided a rough outline of their physiological or biochemical peculiarities: its current status may be ascertained by consulting Barth and Barth (1954), Gregg (1957), Gregg and Kahlbrock (1957), Gregg and Ray (1957) and Gregg (1960).

METHODS

Embryological

Developing embryos were obtained by stripping eggs from pituitary-activated *R. pipiens* females into suspensions of active *R. pipiens* or *R. sylvatica* sperm. After not more than one hour, the developing eggs were separated into small groups of 20–40 members each, and distributed into several large fingerbowls. Each fingerbowl contained 100–200 ml. of 10% amphibian Ringer's solution,

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without phosphate or bicarbonate. Development was allowed to proceed at 10° C., the medium being renewed every two days or so. Immediately before using them as experimental subjects, the embryos were freed of their jelly-coats with the aid of jeweler's forceps.

Chemical

Anaerobiosis was obtained with the help of the apparatus depicted in Figure 1. Twenty jelly-free embryos, along with enough rearing-medium to make a total volume of 2 ml., were placed in each of several 25-ml. Erlenmeyer flasks. The ground glass neck of each flask was closed with a No. 1 two-hole rubber stopper bearing glass inlet and outlet tubes. The flasks were set in the clips of a Dubnof shaking bath running at 24° C., and connected in series with short lengths of rubber tubing. The shaking mechanism was then set in motion at a rate of 85 cycles per minute. At time zero, a source of washed 95% N_2: 5% CO_2 was connected to the inlet tube of the first flask, and gassing was allowed to proceed at a rate of one liter per minute for the duration of the experiment.

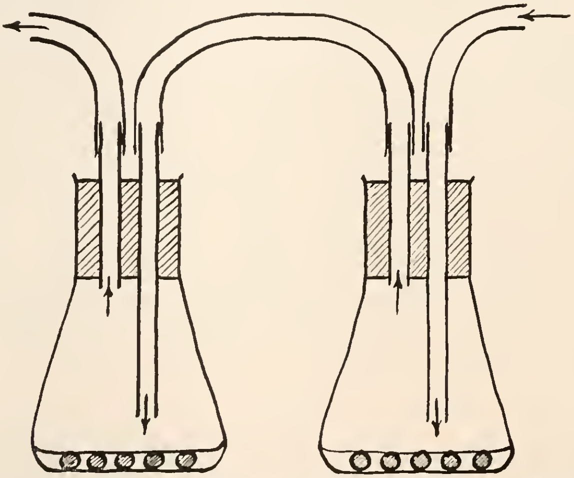

FIGURE 1. Apparatus used to obtain anaerobic embryos. See description in section on chemical methods. Arrows indicate direction of gas flow.

At the ends of chosen intervals, flasks were removed from the distal end of the train and their contents were emptied into 12-ml. graduated centrifuge tubes, each containing 0.5 ml. of 30% trichloracetic acid. The volumes were adjusted with 6% trichloracetic acid to values depending on the expected amounts of lactic acid, and the tubes were placed in a Deepfreeze. After freezing and thawing, the embryos were homogenized with a ball-tipped glass rod, and protein-free extracts were obtained by centrifuging.

The amounts of lactic acid in the protein-free extracts were estimated by a modification of the method of Barker and Summerson (1941). One-ml. aliquots of protein-free extract were treated with equal volumes of 2.5% $CuSO_4 \cdot 5H_2O$ and

125-mg. portions of $Ca(OH)_2$ to remove interfering substances. After centrifuging, 0.5-ml. aliquots of supernatant were combined with 3-ml. aliquots of concentrated sulfuric acid-copper reagent and heated to convert the lactic acid to acetaldehyde. Polyindophenol reagent was added, and the resulting color intensities were read at 560 mμ with the help of a Beckman Spectronic colorimeter. Standards were prepared with lithium lactate.

Terminological

Developmental stages were determined by reference to the data of Shumway (1940), which standardize the course of *R. pipiens* development at 18° C. Regardless of their actual temperature histories, embryos in a given Shumway stage have been assigned the corresponding standard age in hours. Hybrid embryos, even after the curtailment of morphogenesis at Stage 10, have been assigned the same stages as *R. pipiens* control embryos of the same female parentage.

RESULTS

(1) Aerobic embryos contain negligible amounts of lactic acid.

This finding was established directly by a few analyses of *R. pipiens* embryos reared under the conditions described in the section on embryological methods. The results are summarized below:

Clutch	Stage	Standard age	Lactic acid, μg. per embryo
B	3	3.5	0
A	9	21	0.06
A	12	42	0
C	19	118	0.15
C	20	140	0.20

A similar result is implicit in the data presented in Figure 2 and Figure 3, where the values for lactic acid production under anaerobiosis extrapolate satisfactorily to a value of zero at time zero.

These results are in contrast to those of Lennerstrand (1933) and Brachet (1934), who reported much higher aerobic lactic acid values. Barth (1946), however, was able to show that the partial anaerobiosis induced by crowding will result in the production of large amounts of lactic acid, and both he and Cohen (1954) showed that when care is taken to keep embryos well-aerated, there is little or no lactic acid produced. It may be, therefore, that the embryos of Lennerstrand and Brachet were not reared under strictly aerobic conditions.

(2) Embryos begin to produce lactic acid as soon as they are deprived of oxygen.

This result is clearly established by the data of Figure 2 and Figure 3. There is no indication of a lag in the onset of glycolysis of the sort mentioned by Cohen (1954). But the apparent discrepancy between his results and ours may be resolved by considering the methods used to induce anaerobiosis. In our experiments, the embryos were gassed while spread out in a very thin layer of medium over a relatively large surface; therefore, there was probably little delay in the establishment of anaerobiosis. Cohen's embryos, on the other hand, were gassed in a thicker layer of medium spread over a much smaller surface, and anaerobiosis may have developed more slowly. Indeed, Cohen noted that the period of lag was reduced by increasing the number of embryos per volume of medium: on our interpretation, this is to be expected. Thus, Cohen may be regarded as having shown not a lag in the production of lactic acid after anaerobiosis is attained, but merely a lag in the attainment of anaerobiosis.

(3) For at least four hours of anaerobiosis, embryos younger than 72 hours produce lactic acid at constant rates, whereas the rates of lactic acid production by older embryos under similar conditions are decreasing functions of time (Fig. 2, Fig. 3). But, during the first hour of anaerobiosis, embryos of any age glycolyze at rates which for all practical purposes may be regarded as constant (Fig. 3). The latter result has been obtained in experiments on three clutches of control embryos and two clutches of hybrids, other than that represented by Figure 3.

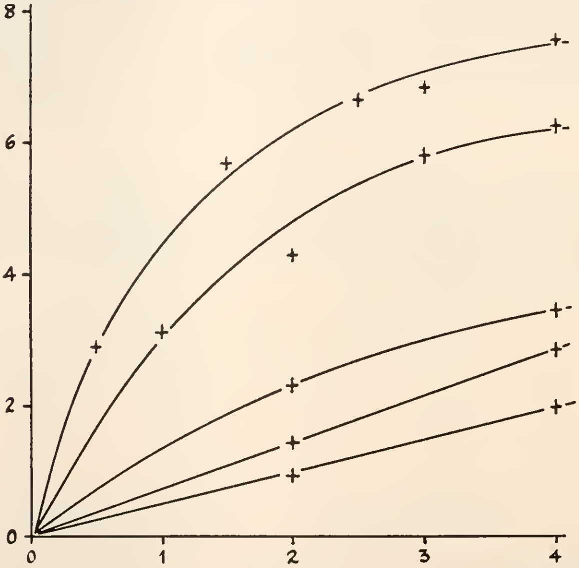

FIGURE 2. Time course of lactic acid production by anaerobic *R. pipiens* embryos, Clutch C. Reading from below to above: Stage 2⁺, standard age 2 hours; Stages 11½ and 15⁻, standard ages 38 hours and 66 hours; Stage 18, standard age 96 hours; Stage 19, standard age 118 hours; Stage 20, standard age 140 hours. Abscissa, time in hours. Ordinate, μg. lactic acid per embryo. (Two points near (0, 0) have been omitted. See table in discussion of result (1).)

Among other things, these findings mean that glycolytic rates obtained by estimating the lactic acid contents of post-neurulae at the beginning and at the end of a long period of anaerobiosis (periods of lengths up to 30 hours are not untypical of the sparse literature on the subject) are not strictly comparable with those similarly obtained on pre-neurulae and neurulae. In establishing the data for Figure 4, we have avoided misleading comparisons by taking the rates of glycolysis to be those exhibited during the first hour of anaerobiosis.

(4) The rate of glycolysis of *R. pipiens* embryos is an increasing function of age. The graph relating age to glycolytic rate (Fig. 4) shows that the capacity to glycolyze develops in two phases. The first phase, spanning the interval between fertilization and the onset of neural fold formation, is characterized by a constant rate of glycolysis. The second phase, from the onset of neural fold formation to hatching, is characterized by an exponentially increasing rate of glycolysis. More precisely:

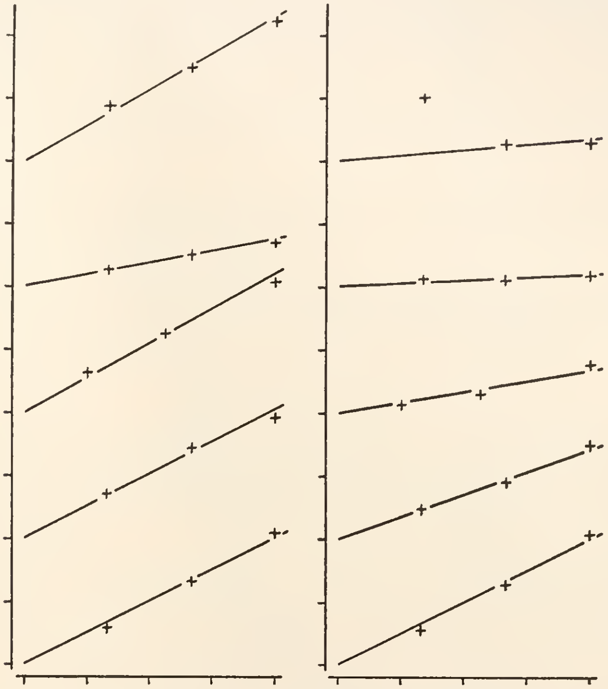

FIGURE 3. Time course of lactic acid production by anaerobic hybrid embryos (right-hand section) and normal control embryos (left-hand section), Clutch F. Reading either section from below to above: Stage 3½, standard age 4 hours; Stage 10½, standard age 30 hours; Stage 13¾, standard age 59 hours; Stage 17½, standard age 90 hours; Stage 19½, standard age 129 hours. Abscissal units lower three curves either section, hours; upper two curves either section, quarter-hours. Ordinal units either section, μg. lactic acid per embryo. (Rates of glycolysis determined from these curves are plotted in Figure 4.)

$$g(t) = 0.44 \qquad (0 \leqq t \leqq 59)$$
$$g(t) = 0.44\, e^{0.0225(t-59)} \qquad (59 \leqq t \leqq 140)$$

where t is the standard age in hours and $g(t)$ is the glycolytic rate in μg. lactic acid per embryo per hour.

It is interesting to note that the acceleratory change in glycolytic rate at 59 hours is paralleled by a similar change in respiratory acceleration at 56 hours (Gregg, 1960.) The close temporal coincidence of the two changes suggests a structural connection of some sort, but details are not yet available.

Comparison with the results of Lennerstrand (1933) and Brachet (1934) is made difficult by complications mentioned in the discussion of results (1) and (3). The data more or less agree with the more circumscribed ones of Barth (1946). But they are clearly inconsistent with those of Cohen (1954), who found that the glycolytic activity of pre-neurulae is a linearly increasing function of age, not a constant function. There is no obvious way to resolve the discrepancy; and, pending further investigation, the two sets of results must remain irreconciled.

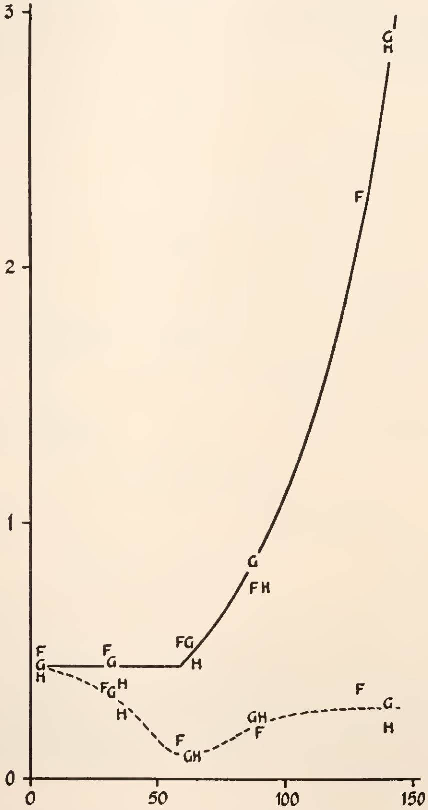

FIGURE 4. Rate of lactic acid production as a function of age, Clutches F, G, H. Lower curve, hybrid embryos. Upper curve, normal control embryos. Abscissa, standard age in hours. Ordinate, μg. lactic acid per embryo per hour. The three points at 4 hours represent both normal and hybrid embryos, whose glycolytic rates at this age are indistinguishable.

(5) Developing hybrid embryos undergo changes of glycolytic rate that are difficult to characterize in any simple way as a mathematical function of age (Fig. 4). Roughly speaking, their capacity to produce lactic acid under anaerobiosis declines from the control value at fertilization to about a fifth of the control value at 59 hours, and then rises slowly until, at 140 hours, the glycolytic rate is about one-eleventh of the control rate. The acceleration at 59 hours may be of the same sort as that in control embryos, but of reduced magnitude. There is no corresponding respiratory acceleration (Barth, 1946). Briefly, our results agree

with those of Barth in showing that hybrid embryos are unable to generate energy by anaerobic glycolysis except at increasingly sub-normal rates. The reasons why are yet unknown.

SUMMARY

1. *R. pipiens* embryos, and gastrula-arrested hybrid embryos obtained by fertilizing *R. pipiens* eggs with *R. sylvatica* sperm, begin to produce lactic acid, without initial lag, as soon as they are deprived of oxygen.

2. Provided that they are younger than about 72 hours (18° C.), embryos of both types are able to sustain the initial rate of glycolysis for at least four hours. Older embryos of both types exhibit a linear production of lactic acid for at least one hour.

3. The development of glycolytic capacity in *R. pipiens* embryos occurs in two phases: one of constant glycolytic rate (0–59 hrs.), the other of exponentially increasing glycolytic rate (59–140 hrs.).

4. The glycolytic rates of hybrid embryos decline from the normal control value at fertilization to about one-fifth of the control value at 59 hours, then increase to about one-eleventh of the control value at 140 hours.

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SPECTRAL SENSITIVITY AND PHOTOTAXIS IN THE OPOSSSUM SHRIMP, NEOMYSIS AMERICANA SMITH [1, 2]

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Few studies have been conducted on photoreception in the Mysidacea. Hess (1910) and Beeton (1959) have both worked on the spectral sensitivity of mysids, but neither has subjected animals to various colors of the spectrum while controlling the intensity of light.

There is no information available in the literature on photoreception in *Neomysis americana*. Hulburt (1957) has shown that their vertical distribution in Delaware Bay is a direct result of light intensity.

This report presents methods and results of laboratory studies on spectral sensitivity and phototaxis in the opossum shrimp, *Neomysis americana* Smith.

The results of a complementary study of the vertical migration of this animal in Narragansett Bay, Rhode Island, will be reported in a separate publication. For purposes of this paper it is sufficient to state that the pattern of vertical migration was similar to that described by Cushing (1951) as typical of many zooplankton species: (1) ascent towards the surface from the day-depth, (2) departure from the surface at or before midnight, (3) return to the surface just before dawn, and (4) sharp descent to the variable day-depth when sunlight starts to penetrate the water. Light was found to be the most important single factor responsible for the migration of these animals.

Materials and Methods

Spectral sensitivity

Although there are many references to the effect of different parts of the spectrum on animal distribution, few have been made where light intensity has been controlled. In order to study the effects of various parts of the spectrum on *N. americana,* it was necessary to keep the light intensity uniform. The procedure adopted was to calibrate a model #846 Weston photronic cell against an Eppley thermopile. The thermopile measures light as a linear function, and therefore is equally sensitive to energy from all parts of the spectrum.

With calibration complete, it was possible to use the photoelectric cell to measure light intensity. The calibration was carried out at the Eppley Laboratories, Newport, Rhode Island, with the following equipment: a Leeds and Northrup model 9835-A stabilized DC microvolt amplifier, a Tinsley photocell

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[2] This study was aided by the Office of Naval Research, Contract NR 104-100.

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galvanometer amplifier type 5214, a Kipp model AL-1 portable galvanometer, an Eppley thermopile #2427 (8 junction, Bi-AS circular, lampblack receiver of sensitivity 0.111 mμ, an Eppley microvolt comparator, and a #846 Weston photronic cell.

The photocell and the thermopile were exposed in turn to the radiant flux through Corning narrow-band interference filters and the outputs compared. The energy intensities of the different light beams were then equalized, as far as possible, with Wratten gelatin neutral density filters, and photocell outputs were determined to insure approximate equality of energy flux.

TABLE I

Calibration of photometer with Corning glass color filters

Filter	Photometer μv.	Thermopile μv.	Energy μv. cm.$^{-2}$	Photometer sensitivity μv. μw. cm.$^{-2}$
Corning				
1–02 (546–559 mμ)	360	.37	3.3	109
1–05 (515 mμ)	500	.63	5.7	88
5–77 (610 mμ)	360	.51	4.6	78
5–75 (460 mμ)	40	.43	3.9	10.1

The photocell was used with a 200-ohm resistor across its terminals and the voltage drop across the resistor was read as the photometer output.

The source of radiant energy was a Westinghouse 150-watt tungsten flood lamp maintained at 110 volts plus or minus 0.5 per cent throughout. The results of the calibration may be seen in Tables I and II.

TABLE II

Calibration of photometer with Corning glass color filters showing equalization of intensity

Filter	Photometer μv.	Thermopile μv.	Energy μv. cm.$^{-2}$	Photometer sensitivity μv. μw. cm.$^{-2}$
Corning				
1–02 with Pyrex	315	.32	2.9	108
1–05 with Wratten filter .3	260	.31	2.9	84
5–77 with Wratten filter .2	147	.30	2.7	54
5–75 with Wratten filter .1	32	.30	2.7	11.8

The thermopile outputs were read with an accuracy of 5%. The calibration of the reference thermopile reproduces the International Pyroheliometric Scale of radiation to about 1%. The photocell outputs were read to within 1%. The precision of the above readings (*i.e.*, repeatability) is better than 5%.

Recalibration of the photocell at the Eppley Laboratories after completion of the experiments showed that no significant changes in energy had taken place during the course of the experiments, and therefore equal light intensity had been maintained.

Spectral sensitivity of animals

A special aquarium, 25″ × 18″, was constructed with a depth of 10 inches. The bottom of the aquarium was of one-quarter-inch plate glass, making it possible to measure light at the bottom of the aquarium. One side was also one-quarter-inch plate glass, to permit observation of the animals during the experiment. This side of the aquarium was covered with a cloth that excluded all extraneous light and served as a hood for the observer during experiments.

The wooden lid of the aquarium included a circular piece 15 inches in diameter, through which holes had been cut in a circular pattern, for the insertion of color filters. With this arrangement, the experimental lamp (centered above the wheel at a height of 15⅛ inches and regulated at 110 volts with a variable transformer) projected down into the water a circular pattern of colored beams, permitting the animals to choose between any of the colored beams and darkness. The wheel was movable and the light beams could be rotated.

Four color filters were used: red, blue, and two shades of green (Table I). Unfortunately, at the time, it was not possible to utilize other filters which transmitted an accurately measurable amount of energy under the experimental conditions. To avoid confusion, green filter 105 (515 mμ) will be referred to as blue-green and green filter 102 (546–559 mμ) as green.

The experiments were conducted at a constant temperature of 19° C. and usually 70–75 animals were placed in the aquarium. After the mysids had remained in darkness for measured periods of time, the experimental light was turned on and every 30 seconds, for ten minutes, the number of individuals congregated in each beam of colored light was counted. Usually another 10-minute experiment was conducted directly after this, wherein the neutral density filters were removed from the three filters containing the lowest numbers of mysids, thereby increasing the energy passing through these three filters. In this way it was possible to observe whether the animals changed their behavior pattern when intensities were greater.

During the course of the experiments, the uniformity of the light intensity was checked by measurement with a Leeds and Northrup K2 potentiometer and the Weston photronic cell.

Phototaxis

Phototactic response of *N. americana* was studied by the method employed by Beeton (1959) in his observations of *Mysis relicta*. Six individuals were placed in a 24-inch glass tube of one-inch diameter, lying horizontally to eliminate any gravitational effects. The experimental light, a 7C7 General Electric lamp, was suspended one foot above the midpoint of the tube (for spectral distribution of lamp, see Beeton, 1959; p. 205, Fig. 1). "After the mysids had been subjected for measured intervals to total darkness or light, one-half of the tube was shaded and the number of mysids in the unshaded half of the tube were recorded at 30-second intervals for a five-minute period. First the right and then the left half of the tube was shaded to detect any bias in the mysid distribution. Control runs with neither half being shaded were made at frequent intervals," Beeton (1959, p. 206).

Biological clocks

Experiments were conducted to determine whether or not *N. americana* would continue to migrate if light stimulation was removed. The observations were made in Plexiglas tubes with an inside diameter of 7.6 cm. Tubes of two lengths were used (one meter and one-half meter) and these could be joined together to obtain greater depth with the use of "O" rings and brass ring nuts and bolts.

Mysids were kept in the dark at a constant temperature and observed around the period of sunset to determine whether or not they would rise to the surface of the water in the tube, as would be expected if a biological time clock were functioning. The animals were viewed through a U. S. Army snooper scope using an infra-red light source (Baylor, 1959). Preliminary observations indicated that mysids were quite insensitive to the red region of the spectrum and therefore the important prerequisite that the experimental animal be unaffected by the light source could be fulfilled.

RESULTS

Spectral sensitivity

In each 30-second interval the total number of mysids counted in all four color beams averaged 10 to 15, the rest remaining in the darkened portion of the tank or in the periphery of the light beams. Only those considered to be within beams were counted. Of those animals which were photopositive, a significant number stationed themselves in the blue-green light beam, the animals in this beam usually outnumbering those in the next most densely occupied beam by approximately 2 to 1 (Table III). Fewer mysids were attracted to light passing through the green and the blue filters, while the red beam attracted the least number of animals. The same order and ratio prevailed regardless of the position of the projected beams in the tank; even as the wheel containing the filters was rotated, mysids could be seen following their respective beams. The neutral density filters were then removed from the green, blue, and red color filters, increasing the energy of these beams above that of the blue-green (see Tables I and II). When this was done, the same 2 to 1 preference for the blue-green was maintained, although there was a slight increase in numbers in the blue beam and a further decrease in numbers in the red.

The Kolmogorov-Smirnov one-sample test was used to determine the significance of the results. This is a test of goodness of fit and is concerned with the degree of agreement between the distribution of a set of sample values (observed scores) and some specified theoretical distribution. It determines whether the scores in the sample can reasonably be thought to have come from a population having a theoretical distribution (Siegel, 1956). This nonparametric technique was selected because it is more powerful than the Chi Square test when there is a continuous variable and the sample is small. D values represent maximum deviation, and in each experiment they show that the distribution of animals was non-random and that the animals showed significant preferences for different colors.

Reaction towards the colored lights remained the same regardless of the time of day or the number of hours the mysids were kept in the dark. In experimental runs in which animals were kept in the dark for over 12 hours, few could be seen

TABLE III

Spectral sensitivity of N. americana

EST Time	Time in dark (hr.)	Number counted in each 10-min. period:				Total # mysids in tank	5% level D
		Red (610 mμ)	Blue (460 mμ)	Green (546–559 mμ)	Blue-green (515 mμ)		
0930	1/2	12	29	28	71	75	.257
*		9	35	32	69	75	.221
1200	3/4	11	26	34	72	75	.258
*		8	32	30	76	75	.226
1022	1	19	31	49	78	75	.215
*		10	41	43	76	75	.200
1015	1 1/4	17	37	40	75	73	.195
*		10	36	35	79	73	.244
1415	1 1/4	35	44	36	81	75	.163
1535	1 1/2	27	43	46	71	71	.144
*		13	52	44	87	71	.194
1607	1 3/4	45	59	61	107	73	.142
2120	2	21	38	44	99	72	.240
*		12	39	41	99	72	.272
1230	2 1/2	15	35	36	70	71	.198
*		11	47	47	75	71	.189
1423	2 3/4	20	27	35	65	70	.193
1543	4	35	46	58	104	72	.178
2125	5	35	58	48	94	69	.150
1023	6	29	52	50	93	74	.120
*		11	42	48	92	74	.227
1642	7	24	30	41	102	67	.264
*		17	56	37	88	67	.189
2215	8	13	23	34	67	46	.233
*		5	34	29	65	46	.233
0906	10 1/4	16	32	31	64	41	.203
*		8	30	28	66	41	.250
0853	11 1/4	36	47	51	88	65	.148
1234	14	24	44	50	81	62	.161
1113	17 1/4	16	28	29	49	70	.151
1523	18 1/2	24	34	33	53	60	.117
0943	21 3/4	15	28	24	55	48	.200
1757	22 1/4	25	34	26	61	69	.168
1602	26	18	33	45	81	53	.209
*		11	36	28	78	53	.255
1100	42 1/2	9	20	19	53	40	.168

* Indicates experiments conducted with neutral density filters removed from red, blue, and green color filters.

in the light beams for the first three minutes, indicating good agreement with phototactic experiments. It is also interesting to note that copepods, which were present in the tank as food for the mysids, exhibited generally the same behavior towards the color beams in both types of experiments.

When unfiltered light from the experimental lamp was permitted to enter the water, all of the mysids in the colored beams were attracted to this white light of much greater intensity. If, however, this white light was then reduced to 4.5

microwatts by interposing neutral density filters, the animals again showed a preference for the blue-green beam, despite the fact that the energy of the white light was greater.

TABLE IV

Phototactic response of N. americana *after periods in light and total darkness*

EST Time	Dark exposure (hrs.)	Light exposure (hrs.)	Numbers in tube		Chi Square
			Shaded	Unshaded	
1253		¼	22	38	4.27*
2148		¼	13	47	19.27*
1347		¾	13	47	19.27*
1500		1	16	44	13.06*
1555		1	8	52	32.36*
1314	1		21	39	5.40*
2110	1¼		6	54	38.40*
1629	2		12	48	21.60*
1945	3		23	37	3.27
1122		3	15	45	15.00*
2301	3¼		31	29	.067
1944	4		26	34	1.067
2400	4		34	26	1.067
1917	5½		18	42	9.60*
1437	5¾		35	25	1.67
2136	8¼		9	41	20.48*
0856	11½		30	30	.000
2259	12¼		38	22	4.27*
1116	13		32	18	3.91*
2206	13		32	18	3.91*
0850	14¼		39	21	5.40*
1343	15¾		31	19	2.88
0900		17	9	31	12.10*
0902	22		38	22	4.27*
1111	23½		31	29	.067
1405	23½		33	27	.900
2145	45½		31	29	.067
Control runs					
0859			30	30	.000
1117			30	30	.000
1124			30	30	.000
1320			33	27	.900
1945			35	25	1.67
1952			29	31	.067
2116			35	25	1.67
2142			31	29	.067
2211			28	32	.13
2307			37	23	3.27
0013			28	32	.13

* Indicates significant Chi Square values at 5% level. Controls were run as frequently as possible after each experiment.

Phototaxis

Six mysids were placed in the horizontal tube and subjected for measured intervals to total darkness or light. One-half of the tube was shaded and the numbers of mysids in the unshaded half were recorded at 30-second intervals for a five-minute period.

Significant differences in distribution were never found in control runs, but were found when one-half of the tube was shaded after the mysids had been in light or dark for a period of time. Mysids were photopositive unless they had been subjected to total darkness for 12 hours; after longer periods in the dark they were photonegative (Table IV). The photonegative condition lasted only for a short time, as they became light-adapted within 3 to 5 minutes of exposure to light. Beeton (1959) found in his laboratory experiments that *Mysis relicta* could be photonegative in the morning and also in the evening, and he stated that it was not likely that the photic response had a persistent diurnal rhythm. The same is true of *N. americana,* since in both the evening and the morning it could become photonegative if kept in the dark for over 12 hours.

Experiments also revealed that mysids which were photopositive could be made to move into the shaded area of the tube if the intensity of the light was increased. This agrees with the results obtained by other workers (Johnson, 1938; Beeton, 1959).

Biological clocks

Examination of mysids kept in total darkness in Plexiglas tubes revealed no significant movement towards the surface at the time of day when the animals in the Bay were ascending. Usually the mysids remained equally distributed throughout the tube at all times.

Discussion

In spectral sensitivity experimentation on mysids, the experimental animals have not previously been offered a choice of lights of different wave-lengths of the same intensity. Hess (1910) worked with *Mesopodopsis slabberi* and found that if these mysids were kept for a time in the dark, and then brought into the light, all of the animals swam rapidly towards the source of the light. When a spectrum was passed through the tank, they rapidly congregated in the yellow-green region and remained there. Since the relative intensities of the different parts of the spectrum were neither controlled nor measured, the animals' apparent preference for the yellow-green may have been due to differences in intensity.

Beeton (1959), experimenting with *Mysis relicta,* measured the response of animals in an aquarium to an experimental light which was passed through different combinations of Corning glass color filters and neutral density filters. He mathematically calculated the total energy output of each filter combination, using the per cent transmission of the color filter and the distribution curve of spectral energy of the experimental lamp. He determined that *M. relicta* showed greatest sensitivity at wave-lengths in the vicinity of 515 mμ and 395 mμ.

Results of spectral sensitivity experiments on *N. americana* indicate a distinct preference for light having a wave-length of 515 mμ. The yellow-green light which attracted Hess' *Mesopodopsis* is closely approximated by the green filter 102 (546–559 $m\mu$), but *N. americana* showed no preference for this under controlled intensity conditions.

The experiments were conducted at a temperature of 19° C. and it would have been desirable to repeat them at lower temperatures, since 19° C. is near the annual maximum in Narragansett Bay.

The comparatively high temperatures may have been one of the reasons why relatively few of the mysids in the experimental tank were attracted to the light beam. On the other hand, the field results of this study indicate that not all of the mysids undergo vertical migration, some animals remaining on the bottom during the night throughout the year. Thus there must be other physiological mechansims operating both in the field and in the laboratory, which are responsible for keeping certain members of the population from responding to light by migrating vertically.

In the spectral sensitivity experiments it was shown that mysids still preferred the blue-green light beam even when the intensity of the other colored light beams was greater. *N. americana* is capable of distinguishing between colors differing in wave-band maxima by only 31–35 $m\mu$, and shows a distinct preference for one of these. Even where greater intensity is present, the mysids seek out this blue-green light. When unfiltered light from the experimental lamp was passed into the aquarium, the mysids quickly congregated in this light, deserting all of the color beams. However, when this light was adjusted with Wratten neutral density filters (4.5 microwatts $cm.^{-2}$), reducing its intensity to a level which was still above that of the colored beams, the mysids again showed a preference for the blue-green. According to John Roche of the Eppley Laboratories (personal communication), when the unfiltered light from the experimental lamp was projected into the tank, more light of the wave-length 515 $m\mu$ was present in this beam than in the blue-green beam, but when this white light was reduced with neutral density filters, less light of 515 $m\mu$ was present in the white beam than in the blue-green beam. In each case the mysids congregated in that beam transmitting the greatest amount of energy of the wave-length 515 $m\mu$.

Experiments indicate that it is not likely that the photic response in *N. americana* is governed by a biological time clock. Experiments also revealed that 12 hours in continuous darkness are required to make this species photonegative. The significance of these findings in regard to the vertical migration of *N. americana* will be discussed in a subsequent publication.

Dr. David M. Pratt reviewed the manuscript and Mr. Theodore A. Napora gave valuable assistance during the experiments. Calibration of equipment and test runs of the entire experimental apparatus were conducted at the Eppley Laboratories, Newport, Rhode Island.

SUMMARY

1. Laboratory experiments were conducted to determine the spectral sensitivity of *N. americana*. The intensity of light beams passing through four Corning glass color filters was made equal with Wratten neutral density filters.

2. The positively phototactic animals showed a definite preference for light passing through a color filter having peak transmission at 515 mμ.

3. Increasing the intensity of light passing through the three other color filters did not alter the mysid preference for the wave-length 515 mμ.

4. Phototactic experiments revealed that *N. americana* was photopositive unless subjected to total darkness for 12 hours; after longer periods in the dark they were photonegative.

5. Experiments indicate that it is not likely that the photic response in *N. americana* is governed by a biological time clock.

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FURTHER STUDIES ON FEEDING AND DIGESTION IN TRICLAD TURBELLARIA

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Previous accounts (Jennings, 1957, 1959) have shown that the triclad Turbellaria feed on a variety of invertebrate animals, such as annelids, molluscs, crustaceans and insect larvae, and that the basis of the feeding mechanism is the protrusible plicate pharynx which is thrust through the integument of the prey to withdraw body contents and pass them back in a finely divided condition into the flatworm gut. The penetration of the prey and the subsequent disruption of its tissues appear to be achieved largely by direct muscular action, but the possibility that this is supplemented by some enzymatic activity has not been investigated, apart from a brief study by Westblad (1922), who failed to find digestive activity in extracts of *Dendrocoelum* or *Polycelis* pharynges. On arrival in the gut the food is phagocytosed by columnar cells of the gastrodermis and digested intracellularly. The sequence of food vacuole formation and intracellular digestion has been described in detail (Willier, Hyman and Rifenburgh, 1925; Jennings, 1957, 1959) but little is known of the enzymes concerned, other than the fact that the food vacuoles contain acid phosphatase and leucine aminopeptidase (Rosenbaum and Rolon, 1960).

In the present investigation two species of triclad Turbellaria, one aquatic and one terrestrial, have been investigated by histochemical methods, to locate and identify any enzymes produced by the pharynx to assist its penetration and disorganization of the food. The course of digestion has been similarly investigated, in each species, in an attempt to identify more of the enzymes concerned and to establish the sequence in which they are produced.

Materials and Methods

The two triclad species examined were *Polycelis cornuta* Schmarda (fresh-water) and *Orthodemus terrestris* (terrestrial). The bulk of the work was carried out on the fresh-water species because of its relative abundance and ease of maintenance in the laboratory.

Flatworms starved for 7 days to clear the gut of traces of previous meals were fed on liver, beef fat or starch paste, the two latter foods being made attractive by mixing with frog blood. The foods were heated to 100° C. and subsequently cooled before being presented to the flatworms, to prevent their inherent enzyme activity being confused with any produced within the flatworm pharynx or gut. The flatworms were fixed at progressive intervals up to 48 hours after an observed meal on one or another of the test foods, and serial sections cut at 8 μ examined for enzyme activity in the pharynx, gut lumen and gastrodermis. Full details of the methods used for fixation, preparation of sections and visualization of enzyme activity have been given in an earlier account of similar studies on digestion in the rhynchocoelan, *Lineus ruber* (Jennings, 1962), and are only summarized here.

Fixation was for 12 hours at 4° C. in 10% formalin buffered to pH 7.0, followed by rapid dehydration in absolute acetone at the same temperature and subsequent embedding in either polyester wax (melting point 37° C.) or paraffin wax (42° C.). When the latter was used, brief clearing in xylol at room temperature was necessary. The polyester technique gave a better histological picture but caused a significant decrease in the demonstrable amount of certain enzymes, notably phosphatases and aminopeptidase, despite the apparent advantage of the lower melting point of the wax, and consequently paraffin wax was used almost exclusively for studies on these particular enzymes. Proteolytic enzymes were demonstrated by the Hess and Pearse (1958) method for endopeptidases of the cathepsin C type (homologous with mammalian chymotrypsin), using as controls incubation media containing cysteine or lead nitrate which act respectively as specific activator or inhibitor, and by the Burstone and Folk (1956) method for exopeptidases of the leucine aminopeptidase type, using heat-inactivated sections as controls. Lipolytic activity was demonstrated after a meal containing beef fat by the Tween 80 method of Gomori (1952), again with heat-inactivated controls. Attempts were made to demonstrate diastatic activity by the Billet and McGee-Russell (1955) method but this gave unsatisfactory results and the presence of carbohydrate-splitting enzymes could only be inferred by tracing progressive digestion of a starch meal by the Lugol's iodine technique. Acid and alkaline phosphatases were visualized by the glycerophosphate methods of Gomori (1952), and controls performed by omitting the substrate from the incubation media and by heat inactivation of sections. The pharynx was examined for possible carbonic anhydrase activity, often associated with production of acid digestive juices, by Hausler's cobalt method (1958), with control sections incubated in the presence of Diamox sodium, a specific inhibitor for this enzyme.

Incubation times at 20° C. and the pH values of the various incubation media can be found in the study on rhynchocoelan digestion referred to earlier.

OBSERVATIONS

The structure of the pharynx and gut

The structure of the triclad pharynx and of the gut and its lining gastrodermis have been described in detail elsewhere (Hyman, 1951; Jennings, 1957). Briefly, in both species investigated here, the pharynx is a highly muscular tube which lies in the pharyngeal chamber in the posterior region of the body. It is directed backwards and can be protruded through the ventral mouth by simple muscular elongation. The pharynx contains along its entire length outer and inner longitudinal and circular muscle layers, a layer of acidophil and basophil gland cells between these, radial muscles and a well developed nerve plexus. The gut proper, in each species, is of the typical triclad pattern with one anterior and two posterior branches, each of which is further subdivided. The gastrodermis consists of a single layer of cells standing on a thin basement membrane and containing only two cell types. The larger and more numerous cells are columnar, 35–40 μ in height, with basal nuclei and granular cytoplasm usually containing phagocytosed food in various stages of digestion. The second type of cell is the "granular club" (Hyman,

1951) or "sphere cell" (Jennings, 1957) and is pear-shaped, 20–30 μ in height, and contains numerous homogeneous spheres which in the fully developed cell are intensely acidophilic and stain strongly with Millon or similar reagents for protein. The spheres within any one cell are always of the same size and appear to mature with the cell. Thus, in small sphere cells the spheres are 1 μ or less in diameter and increase up to 5–6 μ in the mature cell. During prolonged starvation the number of sphere cells decreases, relative to the columnar cells, and the spheres of those persisting show reduced affinity for stains.

Enzymes produced in the pharynx and gut during feeding and digestion

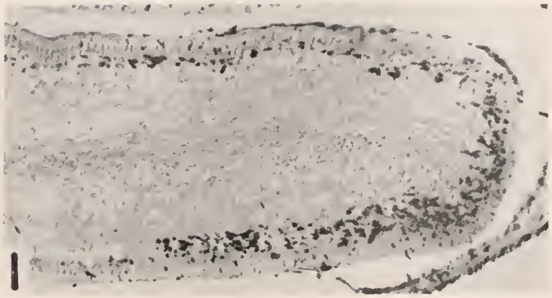
(1) The pharynx

In both *Polycelis* and *Orthodemus* a large proportion of the acidophil gland cells of the pharynx show a strong positive reaction for endopeptidases of the cathepsin C type, particularly around the free distal end (Fig. 1). The glands are flask-shaped and open on to the outer surface, only, of the pharynx (Fig. 2), never into the lumen. Sections of the pharynx prepared immediately after feeding showed that many of these gland cells were discharged and shrunken, and there can be little doubt that their secretions are used to supplement the muscular pressure exerted by the pharynx during the penetration of the prey, by softening or dissolving the tissues of the body wall. The marked concentration of gland cells around the tip of the pharynx supports this conclusion.

Penetration of the prey occupies 30 to 60 seconds and once within it the pharynx moves about and draws up organs, tissues and body fluid. This part of the feeding process may last for several minutes, and again there can be little doubt that break-up of the prey's body contents by the muscular activity of the pharynx is supplemented by proteolysis effected by secretions from the pharyngeal glands. Since these open on to the outer surface of the pharynx and not into the lumen, their secretions are presumably poured into the body cavity of the prey to attack and disrupt its contents whilst tissues already disorganized are being ingested. In this connection it is significant that the pharynx is always inserted *into* the prey, even when the latter is manifestly small enough to be swallowed whole, as when oligochaetes of a smaller diameter than the resting pharynx are captured. In such cases the pharynx, or its distal portion, is extended until it is slim enough to enter the prey in the usual manner (Fig. 3) and so allow the secretions of the pharyngeal glands to attack its contents. This feeding pattern is followed even with test meals of blood or finely chopped liver, when the pharynx enters the food mass and withdraws material from the center rather than merely being applied to the surface layers.

The optimum pH for visualizing the endopeptidase activity was 5.0, and consequently it was thought that the pharynx might produce acid to provide the proteolytic secretions with the necessary working conditions. The enzyme often concerned with production of acid digestive juices is carbonic anhydrase, but no trace of this enzyme could be found in either the *Polycelis* or *Orthodemus* pharynx.

The cytoplasm of the acidophil endopeptidase gland cells shows at all times a weak reaction for acid phosphatase. No other enzyme activity, proteolytic or otherwise, could be detected in the pharynx of either species.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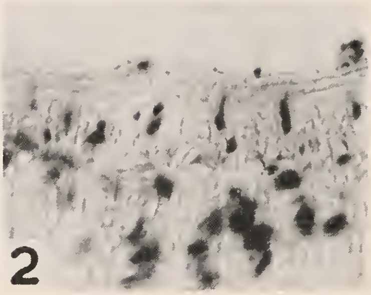
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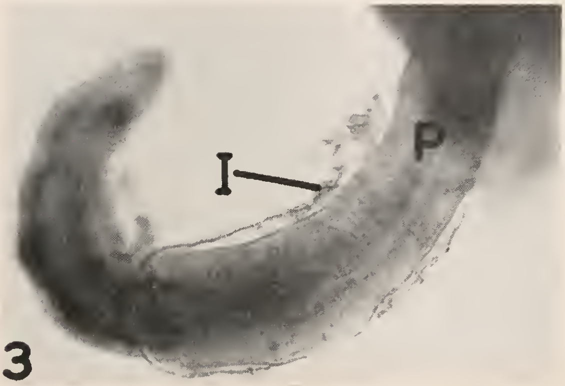

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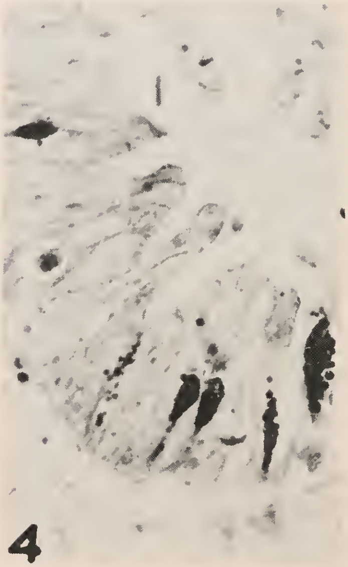
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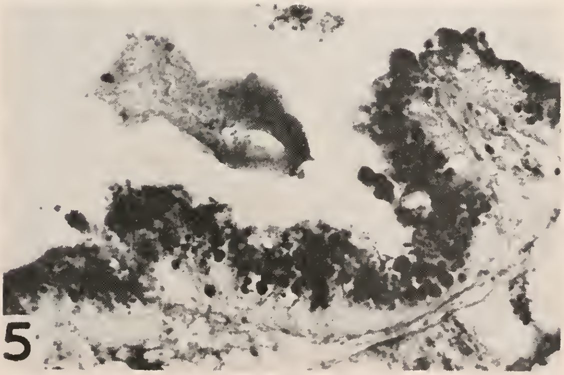
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FIGURE 1. Longitudinal section of the *Polycelis* pharynx, showing the distribution of acidophil endopeptidase-producing gland cells. Hess and Pearse method. The tissue at the extreme bottom right is body wall and epidermis, and the dark bodies seen here are rhabdites which have stained strongly with the eosin counterstain. Scale: 1 cm. = 125 μ.

FIGURE 2. A portion of the outer layers of the *Polycelis* pharynx, showing endopeptidase gland cells discharging on to the outer ciliated epithelium. Hess and Pearse method. Scale: 1 cm. = 25 μ.

FIGURE 3. The *Polycelis* pharynx attacking a small oligochaete. Note that the pharynx (P.) has been inserted *into* the oligochaete and that only the integument (I.) remains outside the pharynx. Unstained whole mount. Scale: 1 cm. = 250 μ.

(2) The gut

Cathepsin C type endopeptidases

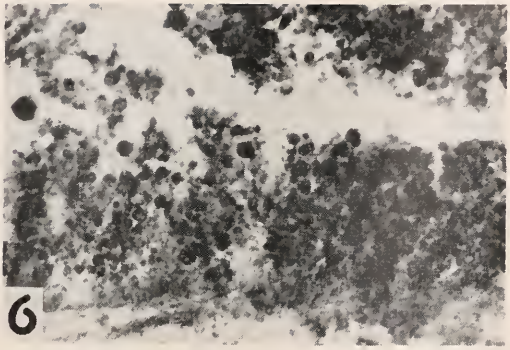
The spheres of the gastrodermal sphere cells show in both species an intense positive reaction for the cathepsin C type endopeptidases (Fig. 4), and a similar reaction is given by fine granules which occur in the cytoplasm of the columnar cells when these are cleared of digesting food by 2 to 3 days' starvation.

Flatworms killed immediately after a meal of boiled liver show faint traces of endopeptidase activity in the material lying in the gut lumen, and this is derived, no doubt, from secretions poured on to the food by the glands of the pharynx before ingestion. The amount of endopeptidase activity in the contents of the lumen increases with time up to a maximum reached 4 hours after feeding (Fig. 5), and during this time there is a decrease in the number of the large and mature sphere cells relative to the number of columnar cells. This decrease in the number of sphere cells is not constant throughout the gastrodermis, however, and some regions may be quite devoid of them whilst others have the normal complement. Usually the disappearance of sphere cells from a region of the gut coincides with the presence of food and the development of maximum endopeptidase activity in that region, but food showing such activity may be found in parts of the gut lined by the normal proportions of sphere and columnar cells. Such situations are probably due to material in the lumen being passed into a region of the gut away from that where the enzyme activity originated, by the convulsive contractions of the flatworm during fixation. Individual spheres of the same size and reaction as those within mature sphere cells are occasionally found either between columnar cells or lying free in the gut lumen. It would appear from this, and the decrease in sphere cell numbers noted above, that mature sphere cells discharge their contents when food enters the gut, and that the lumen endopeptidase activity comes from this source.

The endopeptidase activity developed in the gut lumen does not cause complete homogenization of the food, and even at the peak of its activity, as shown by the intensity of the histochemical reaction, distinctive components of the food, such as erythrocytes, muscle fibers, liver cell nuclei, etc., are often clearly recognizable. The columnar cells of the gastrodermis commence phagocytosis of the food immediately it enters the gut, and the smaller food particles pass rapidly into the cells so that they are not exposed for long to the lumen proteolysis. The function of the latter appears to be primarily to facilitate phagocytosis by softening and breaking up the larger pieces of the food, rather than to render it completely soluble. There appears to be only an initial discharge of endopeptidase when food enters the gut, not a continuous one for as long as it remains in the lumen, and sections prepared at intervals up to 48 hours after feeding show that any food particles too large for phagocytosis which survive this initial discharge persist unchanged until eventually expelled from the gut. This situation is particularly liable to arise if starved flatworms are allowed to

FIGURE 4. The gastrodermis in a *Polycelis* starved for 7 days, showing sphere cells and columnar cells. The sphere cells show an intense positive endopeptidase reaction. Hess and Pearse method. Scale: 1 cm. = 20 μ.

FIGURE 5. Transverse section of a portion of the *Polycelis* gut 4 hours after a meal of boiled liver. Liver lying in the gut lumen (top left) shows a positive endopeptidase reaction, especially the right-hand portion, and the gastrodermis is loaded with phagocytosed liver showing a similar but stronger reaction. Hess and Pearse method. Scale: 1 cm. = 40 μ.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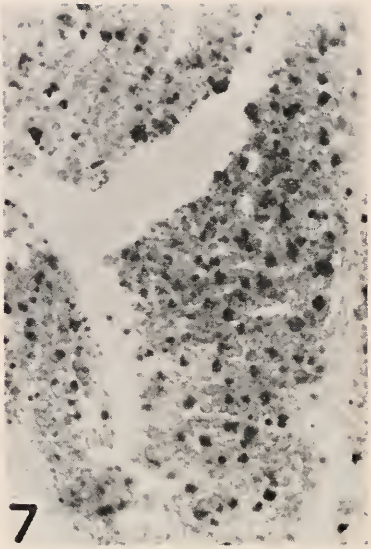
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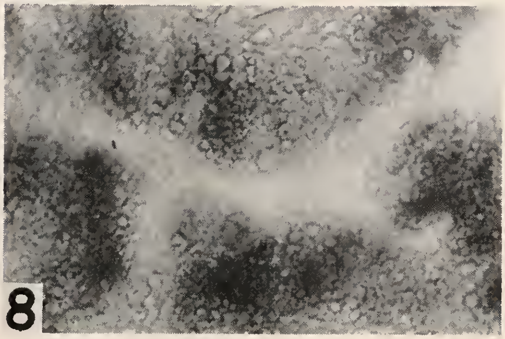
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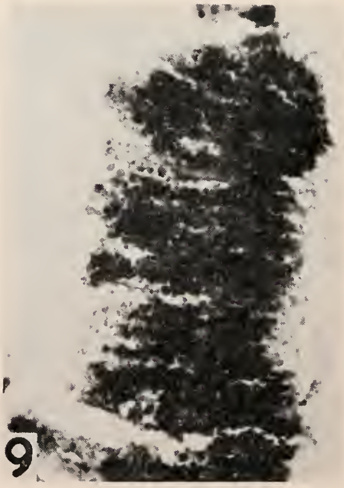
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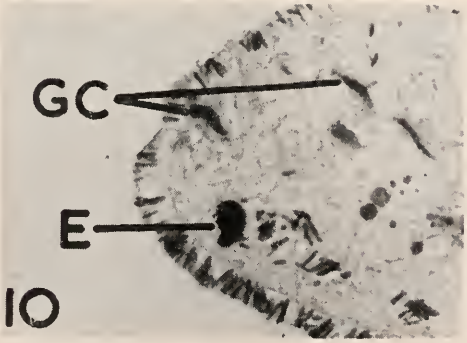
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Figure 6. Transverse section of a portion of the *Orthodemus* gut 12 hours after a meal of boiled liver. The gastrodermis is loaded with food vacuoles, all showing an intense positive reaction for leucine aminopeptidase. Burstone and Folk method. Scale: 1 cm. = 20 μ.

Figure 7. Transverse section of a branch of the *Orthodemus* gut 12 hours after a meal containing a large proportion of fat. Many of the food vacuoles show lipolytic activity, seen here as black spheres or granules. Gomori Tween 80 method. Scale: 1 cm. = 20 μ.

Figure 8. Transverse section of the *Polycelis* gut 4 hours after a meal of boiled liver. The cytoplasm and food vacuoles show acid phosphatase activity (dark areas). Gomori method. Scale: 1 cm. = 40 μ.

Figure 9. Transverse section of the *Orthodemus* gut as in Figure 6 but treated here for alkaline phosphatase. The food vacuoles show intense alkaline phosphatase activity. Gomori method. Scale: 1 cm. = 20 μ.

feed until replete, when they often ingest more food than can be adequately dealt with in the lumen. The persistence of unchanged food elements in the lumen for up to 48 hours after feeding has been interpreted previously as showing the complete absence of intraluminar digestion (Jennings, 1957), but the present demonstration of endopeptidase activity in the contents of the lumen leaves little doubt as to the occurrence of at least a limited amount of intraluminar digestive activity in the two species investigated here.

Food phagocytosed from the lumen continues to show endopeptidase activity within the vacuoles of the columnar cells, and since this increases in intensity as the vacuoles pass back deeper into the cells, endopeptidases must be secreted into the vacuoles from the surrounding cytoplasm, perhaps from the reactive granules so prominent when the cells are cleared of other inclusions. As the vacuoles pass back into the columnar cells, more form distally until the cells are loaded with food undergoing intracellular digestion and showing intense endopeptidase activity (Fig. 5). Eight to 12 hours after feeding, the contents of the vacuoles are reduced to compact homogeneous masses, and the endopeptidase activity fades gradually, first from vacuoles deep within the cells and then from the rest, indicating that the first stage of digestion affecting breakdown of protein to peptones and polypeptides is completed. The food then passes into the second stage of digestion, in which exopeptidases complete proteolysis down to amino acids, and lipases and carbohydrases attack fats and carbohydrates exposed by the digestion of cell walls or other cytoplasmic membranes.

The optimum pH value for visualizing endopeptidase activity in the lumen and gastrodermis of both species was pH 5.0, and this indication that the first stage of digestion is carried on in an acid medium agrees with the results obtained by feeding test foods plus indicators when a pH value of 4.6 was found in the food vacuoles 6 hours after feeding (Jennings, 1957).

Leucine aminopeptidase (exopeptidase)

Leucine aminopeptidase activity is confined to the columnar cells of the gastrodermis in both species and was never found in either the gut lumen or the sphere cells.

As endopeptidase activity fades from the food vacuoles it is gradually replaced by leucine aminopeptidase, supplemented, presumably, by other exopeptidases not demonstrated by the technique used here. The time of onset of the exopeptidase activity varies with the size of the original meal which influences the amount of food phagocytosed by the columnar cells. Thus, when only a small meal is taken, and each columnar cell forms relatively few food vacuoles, exopeptidase activity appears in the latter as early as two hours after feeding, but when a large meal has allowed the columnar cells to become packed with food vacuoles, the activity may not appear for 12 to 18 hours. On the average, endopeptidase activity is replaced by exopeptidase 8 to 12 hours after feeding.

The exopeptidase activity overlaps the endopeptidase, to a degree determined

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FIGURE 10. Longitudinal section through the anterior end of *Polycelis*, showing an eye (E.) and acidophil endopeptidase-producing gland cells (G.C.) in the parenchyma, which discharge through the epidermis. Rhabdites in the latter are stained strongly by the eosin counterstain. Hess and Pearse method. Scale: 1 cm. = 25 μ.

by the amount of food ingested, and recently formed vacuoles in the distal region of the columnar cells may show marked endopeptidase activity whilst those deeper within the cell give a weak exopeptidase reaction. The latter increases in strength with time and appears in more and more of the vacuoles until eventually all food undergoing intracellular digestion gives an intense reaction (Fig. 6). This activity persists for as long as food remains in the gastrodermis and, depending upon the size of the meal taken, may still be present 48 hours after feeding.

Columnar cells of starved flatworms show no reaction for leucine aminopeptidase, and it would appear that exopeptidases, unlike the endopeptidases, are normally present in an inactive form and are not activated until food vacuoles are present.

The optimum pH for visualizing exopeptidase activity was 7.2 in both species, indicating that the second and final stage of proteolysis proceeds in a slightly alkaline medium, as in most other animals.

Lipase

In both species the gastrodermis shows a small amount of lipolytic activity during starvation, and this probably represents the utilization of reserve fat which is laid down in the columnar cells when food is plentiful (Jennings, 1957, 1959).

Both species show lipolytic activity in food vacuoles formed within the columnar cells after a meal containing beef fat (Fig. 7), and this develops as the endopeptidase activity fades. No lipolysis was found in the gut lumen, and pieces of fat too large for phagocytosis lay there quite unchanged until expelled from the gut.

The optimum pH value for demonstrating lipolytic activity was 7.2 in both cases, the same as for the exopeptidase.

Carbohydrases

No conclusive results were obtained from the Billett and McGee-Russell method for β-glucuronidase when applied to sections of flatworms killed at intervals after meals containing boiled starch. Treatment with Lugol's iodine, however, showed progressive conversion and disappearance of the starch within food vacuoles, whilst any remaining in the lumen was quite unchanged. Thus, both *Polycelis* and *Orthodemus* possess diastatic enzymes but these remain as yet unidentified.

Phosphatases

Both acid and alkaline phosphatases occur in the columnar cells of the gastrodermis during intracellular digestion, and there is a marked correlation between the development of endopeptidase and acid phosphatase, in the first stage, and the remaining digestive enzymes and alkaline phosphatase, in the second.

In starved individuals the cytoplasm of the columnar cells shows a weak reaction for acid phosphatase but none for alkaline. As food vacuoles form and endopeptidase activity develops within them, there is a simultaneous but less marked increase in acid phosphatase in both the vacuoles and the surrounding cytoplasm. The peak of acid phosphatase activity coincides with that of the endopeptidase (Fig. 8), but at no time is the reaction particularly intense. This is due, perhaps, to loss of enzyme during preparation of sections, since check sections of mammalian tissue treated in the same way show less than the expected amount of acid phosphatase.

The acid phosphatase activity decreases as the endopeptidases fade from the vacuoles, and is gradually replaced by alkaline phosphatase. This develops in both cytoplasm and vacuoles simultaneously with the leucine aminopeptidase and lipase, and at its peak every vacuole shows a most intense reaction (Fig. 9). The activity persists for as long as food vacuoles are present in the columnar cells.

Neither acid nor alkaline phosphatases are demonstrable in the sphere cells or gut lumen at any stage of digestion.

The pH optima for demonstration of the two phosphatases were, respectively, pH 5.0 (acid) and pH 9.0 (alkaline).

Enzymes in the parenchyma

During starvation, regions of the parenchyma often show a weak reaction for endopeptidase, aminopeptidase and lipase activity. This reflects, no doubt, the utilization of reserve protein and fat which are stored in the parenchyma (Jennings, 1957).

Certain acidophil gland cells which lie in the parenchyma along the anterior margin of the body give a marked endopeptidase reaction (Fig. 10). These glands are of an elongate flask shape and discharge between the epidermal cilia. Their function is unknown, but it is possible that their secretions are passed over the body by ciliary action during locomotion to help in keeping the surface free of microorganisms or to make the flatworm distasteful to would-be predators.

Discussion

The main features of interest emerging from the present study on triclad feeding and digestion are, respectively, the demonstration of proteolytic activity in the acidophil gland cells of the pharynx and the proof that digestion is not exclusively intracellular as was previously believed.

The presence of cathepsin C type endopeptidases (proteases of the type initiating proteolysis) in the pharynx glands, the concentration of such glands around the tip of the pharynx, and the discharged and shrunken appearance of the glands immediately after feeding leave little doubt that proteolytic secretions are used to supplement muscular action in the penetration and subsequent internal disorganization of the prey by the pharynx. The triclads are not unique amongst the Turbellaria in this respect, however, for the acotylean polyclads likewise use proteolytic secretions from the pharynx or gut to supplement the muscular action of the pharynx during pre-ingestion break-up of the food (Jennings, 1957). In their case the pharynx is of the same basic structure as the triclad but is much expanded to form a ruffled curtain—the ruffled plicate pharynx—which is extended over the prey to envelope it and act as an external "stomach," rather than being inserted into it to act as a suction tube.

The belief that digestion in the triclad is exclusively intracellular rested on the fact that recognizable food elements may persist in the lumen for up to 48 hours after feeding, but whilst the present work has confirmed that this does occur, for reasons mentioned in the text, it has also shown beyond reasonable doubt that there is some intraluminar digestion by endopeptidases.

The endopeptidase responsible for intraluminar digestion is produced by the sphere cells of the gastrodermis which in the past have been regarded as protein reserve cells (Hyman, 1951; Jennings, 1957). This conclusion was based on the progressive reduction in the number of sphere cells during starvation, but in view of the undoubted glandular nature of these cells this probably represents a simple regression, such as occurs in the cells of other animal digestive organs during prolonged starvation, rather than the utilization of specific protein reserves.

Intraluminar digestion is followed by phagocytosis and completion of digestion intracellularly by exopeptidases, lipase and carbohydrases. This sequence of events closely resembles that occurring during digestion in the related rhynchocoelan, *Lineus ruber* (Jennings, 1962). In the rhynchocoelan, however, lumen digestion is far more extensive and results in the food being completely homogenized before it enters the gut cells. This is clearly related to the fact that the food is swallowed whole, whereas in the triclad it is already considerably broken up when it reaches the gut, and the bulk of it is immediately available for phagocytosis and intracellular digestion. Consequently there is relatively less intraluminar digestion in the triclad, and what does occur appears to be aimed at reducing the particle size of the food to make it available for phagocytosis, rather than at achieving complete breakdown to simpler substances.

The difference in the amount of intraluminar digestion in the triclad and the rhynchocoelan, itself the result of differences in the respective feeding mechanisms, is reflected in the subsequent intracellular processes. In the rhynchocoelan, food entering the gastrodermis passes almost immediately into the second exopeptidase stage of digestion. In the triclad, food may enter the gastrodermis only slightly affected by the lumen-acting endopeptidase, or even completely unaffected, if phagocytosed soon after the meal, and consequently it must first be attacked by endopeptidases before it is available to the later acting enzymes. As a consequence of this there is far more intracellular endopeptidase activity in the triclad than in the rhynchocoelan. This affords a good demonstration of the effect a particular type of feeding mechanism may have upon subsequent digestive processes.

The two types of phosphatase found in the triclad gut appear to be linked with formation of the intracellular enzymes. Acid phosphatase is closely linked with the first or endopeptidase stage, and Rosenbaum and Rolon (1960) suggest that it may be concerned with food vacuole formation. Alkaline phosphatase is linked with the appearance of the later acting enzymes, and may well be concerned in the release of energy needed for secretion of the various enzymes and the absorption of the products of digestion from the vacuoles.

SUMMARY

1. Feeding and digestion in two species of triclad Turbellaria, one aquatic, the other terrestrial, have been investigated by histochemical methods to locate and identify a selection of the enzymes concerned in the two processes.

2. In both species the pharynx possesses acidophil gland cells which produce endopeptidases of the cathepsin C type, and the available evidence indicates that these are used to assist the pharynx in its penetration of the prey's body wall, and the subsequent disruption of the body contents prior to ingestion.

3. Food entering the gut is attacked by extracellularly-acting endopeptidases, similar to those produced in the pharynx, and originating from the sphere cells of

the gastrodermis. This intraluminar digestion continues and extends break-up of the food initiated by the pharynx, and serves to make the bulk of it available for phagocytosis and intracellular digestion.

4. Columnar cells of the gastrodermis phagocytose food from the gut lumen and digest it within vacuoles containing enzymes secreted from the cytoplasm in a definite sequence.

5. The contents of the food vacuoles are attacked first by endopeptidases similar to those secreted into the gut lumen and acting in an acid medium of pH 5.0.

6. Endopeptidase activity within the vacuoles is eventually replaced by exopeptidases, such as leucine aminopeptidase, plus lipase and unidentified carbohydrases, all acting in a slightly alkaline medium of pH 7.2.

7. Secretion of the various intracellular enzymes involves the appearance of phosphatases in both the cytoplasm and the vacuoles of the columnar cells. Acid phosphatase appears to be concerned with the secretion of endopeptidase in the first stage of intracellular digestion and alkaline phosphatase with the production of the other digestive enzymes.

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THE "HERTWIG EFFECT" IN TELEOST DEVELOPMENT

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In 1911 O. Hertwig found that when frog sperm were treated to prolonged exposures of radium they retained their ability to fertilize eggs but lost their genetic function. The result was similar to parthenogenetic (gynogenetic) development, wherein the egg developed without benefit of sperm chromatin. Since 1911 the study of parthenogenesis produced by this method has been limited, among the vertebrates, almost exclusively to the Amphibia. It is therefore of interest to determine whether the exposure of other vertebrate sperm to ionizing radiations could similarly result in parthenogenetic development.

Materials and Method

Fundulus heteroclitus is a marine teleost common in the Woods Hole area, readily obtained by the Marine Biological Laboratory Supply Department. They are kept in the laboratory in running sea water until used. The method for obtaining eggs and rearing the embryos is that described by Costello *et al.* (1957).

Prior to x-irradiation a concentrated suspension of sperm was prepared by removing the testes from five to six sexually mature males and placing them on a plastic depression plate and macerating them. A portion of this suspension was then removed to another and similar plate to be kept and used as control. The remaining sperm were irradiated in a cesium-137 irradiator at an output of 5000 r/min. The exposures used were 500 r, 1000 r, 5000 r, 50,000 r, 100,000 r, and 150,000 r. Sperm samples were removed at appropriate intervals for the fertilization of normal eggs.

Fertilization was accomplished by placing normal eggs, recently stripped, in a stender dish with a very small volume of filtered sea water, and adding the sperm by means of a glass rod dipped into the appropriate suspension. Between each use the rod was washed in tap water to kill any adherent sperm, and was thoroughly dried. In the earlier series the fertilization occurred immediately after irradiation of each portion of sperm, but since the time required for the highest level of irradiation was brief, all later series were fertilized simultaneously following the completion of all irradiations. It was found that sperm samples added to the normal eggs were all active, showing motility even after 150,000 r.

The developing embryos were raised in fingerbowls containing filtered sea water, to avoid unnecessary contamination. All non-cleaving eggs were removed after the cleaving eggs had attained the blastodisc stage. At the end of the experiment the embryos were again photographed, and fixed in Bouin's solution and

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prepared for possible cytological study according to the method of Costello *et al.* (1957).

EXPERIMENTAL DATA

In all cases, the per cent of eggs which cleaved, following fertilization with x-irradiated sperm, was half or less than that of the control. The per cent cleavage did not necessarily decrease proportionately with increased exposure to the sperm (see Table I). Normal variations in cleavage per cent are such that separate controls of the same eggs were used for each series. As the season progressed fewer eggs were available and fewer of the control eggs developed.

Among those eggs fertilized with irradiated sperm there was no observable lag in the cleavage time for the first division and until the blastula stage, when compared with the controls. This held true for all irradiation levels, even at 150,000 r to the sperm. Following gastrulation (stage #12; see Oppenheimer, 1937; Solberg, 1938; or Rugh, 1962) most eggs fertilized by sperm which had been exposed to 5000 r or more showed some slight retardation over the controls, to the extent of about one full stage of development (Plate I, Figs. 1–8). Those eggs fertilized with sperm receiving the higher exposures of 50,000 r or more showed a slightly greater retardation than those fertilized by sperm which had been irradiated to a lower level. However, the anomalies following fertilization with 5000 r sperm appeared to be more severe than those arising from sperm exposed to higher levels of irradiation. Some retardation was seen in eggs fertilized by 2000 r sperm, and this retardation occurred beginning at stage #14, while the lower exposures delayed the retardation to stage #22. Sperm exposed to 500 r were unable to adversely affect development.

TABLE I

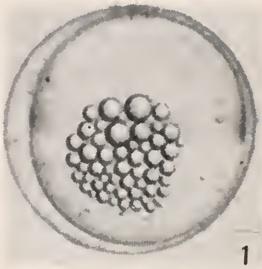
*Percentage of eggs developing (cleaving) after fertilization with x-irradiated sperm**

Exposure (r)	Series 1	Series 2	Series 3
Control–0	84.1 (107)	100 (25)	20 (5)
500 r	29.5 (44)		
1000 r	47.2 (36)		
2000 r	42.0 (50)		
5000 r	47.1 (34)	46.6 (30)	
50,000 r	53.1 (49)	7.1 (28)	20 (10)
100,000 r		3.4 (29)	7.7 (13)
150,000 r			7.1 (14)

* Note: Total number of eggs examined in parentheses.

In eggs fertilized by sperm exposed to 5000 r or 50,000 r, some appeared to develop equally well with the controls in every respect (Plate I, Figs. 9, 10 and Plate II, Figs. 11, 12).

More than half of the embryos developing from eggs fertilized by sperm exposed to 5000 r or more developed pulsating hearts and pigment patterns similar to those of the controls, but all were stunted or otherwise malformed with the exception of the few aforementioned (Plate I, Figs. 5–8, Plate II, Figs. 13–16). Some of these embryos developed corpuscles, and many showed these corpuscles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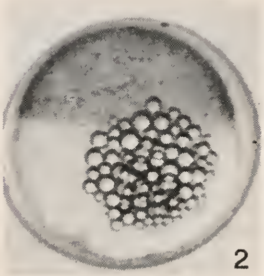
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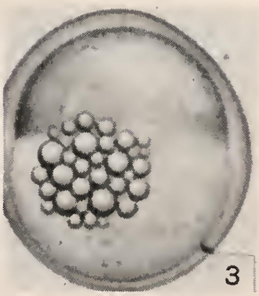
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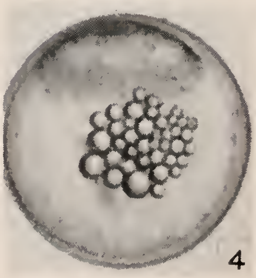
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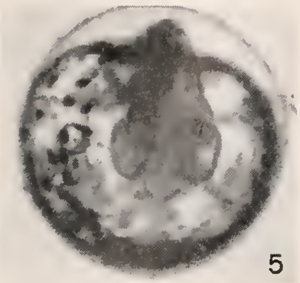
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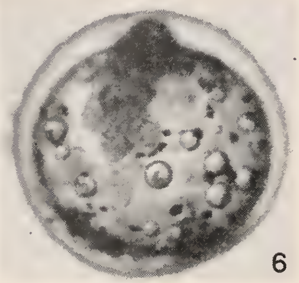
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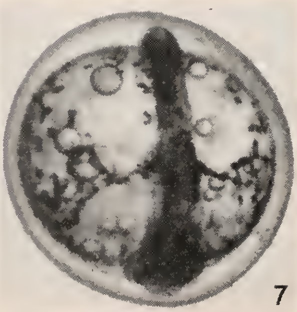
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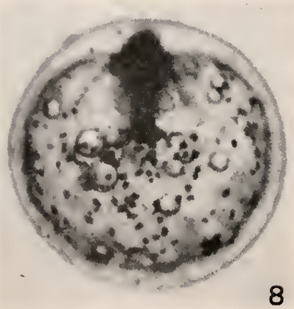
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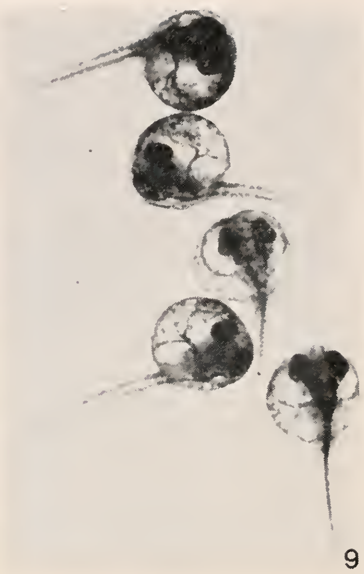
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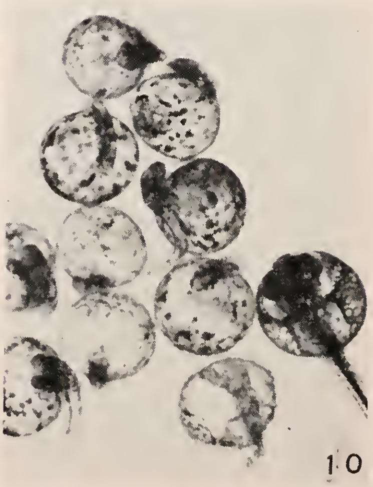
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PLATE I

FIGURES 1–4. All embryos are from the same series and all are one day in development. FIGURE 1. Control, midgastrula. FIGURE 2. From 50,000 r to sperm, early gastrula. FIGURE 3. From 100,000 r to sperm, early gastrula. FIGURE 4. From 150,000 r to sperm, late blastula.

circulating. In all embryos not possessing a pulsating heart, edema developed. The heart beat, even without corpuscles, was found to be of a rate similar to that of the normal controls with their full complement of corpuscles.

One severe abnormality rather common to both the 5000 r and the 50,000 r series was the failure of the embryo to form either a neural or body axis. The blastoderm developed into an amorphous mass of protoplasm devoid of any recognizable structure, but often possessing pigment cells on the surface. Some eggs ceased development at late blastula or early gastrula stages.

DISCUSSION

The work of O. Hertwig (1911), Oppermann (1913), Porter (1939), Rugh (1939) and others has shown that haploid development (either androgenetic or gynogenetic) generally exhibits a specific set of anomalies. Among the Amphibia, haploid development appears to be normal until gastrulation, when a delay in development is noted when comparisons are made with simultaneous controls. Neurulation is even more delayed and abnormal. In older embryos (six to eight days), the brain is poorly differentiated and the circulatory system is non-functional, although heart and corpuscles may have formed. Edema generally appears under such conditions, probably because of the failure in excretory function. Tail formation is retarded, giving the haploids a stunted appearance, accentuated by lordosis. Similar observations have been made by Oppermann (1913) on parthenogenetic trout.

Some of the eggs developing from eggs fertilized by sperm exposed to 50,000 r and all embryos from sperm exposed to higher levels of irradiation showed the same characteristics described above. Since Rugh (1939) found that among the Amphibia, some 90% of the embryos developing from eggs fertilized by sperm exposed to 50,000 r were haploids, it is quite probable that the *Fundulus* embryos which survived the high levels of irradiation and appeared normal were indeed haploids and parthenogenetic at the beginning. This is supported by the fact that the eggs fertilized by sperm which had been exposed to 5000 r were more severely abnormal than any of those which survived fertilization by sperm exposed to either 100,000 r or 150,000 r. It thus appears that exposures of sperm up to 50,000 r are not quite sufficient to completely eliminate the genetic contribution of the sperm in every case.

There was one embryo from the 5000 r series and two from the 50,000 series which were indistinguishable from the controls. These were probably recovered diploid embryos. Tyler (1941) discussed the possible methods of regulation from haploidy to diploidy in the frog. Since there was no evidence of cleavage

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FIGURES 5–8. The same series of embryos seen in Figures 1–4 but 3¼ days after fertilization, each derived from the egg shown to its left. Embryo in Figure 7 is almost normal, possessing circulation comparable to the control. Note poorly formed central nervous system in all but the controls.

FIGURE 9. A group of control eggs 7 days after fertilization. Note uniformity in development.

FIGURE 10. A comparable group of embryos developing from eggs fertilized by sperm exposed to 5000 r x-rays (Cs-137), showing optimum development among this group. One embryo appears to closely resemble the controls.

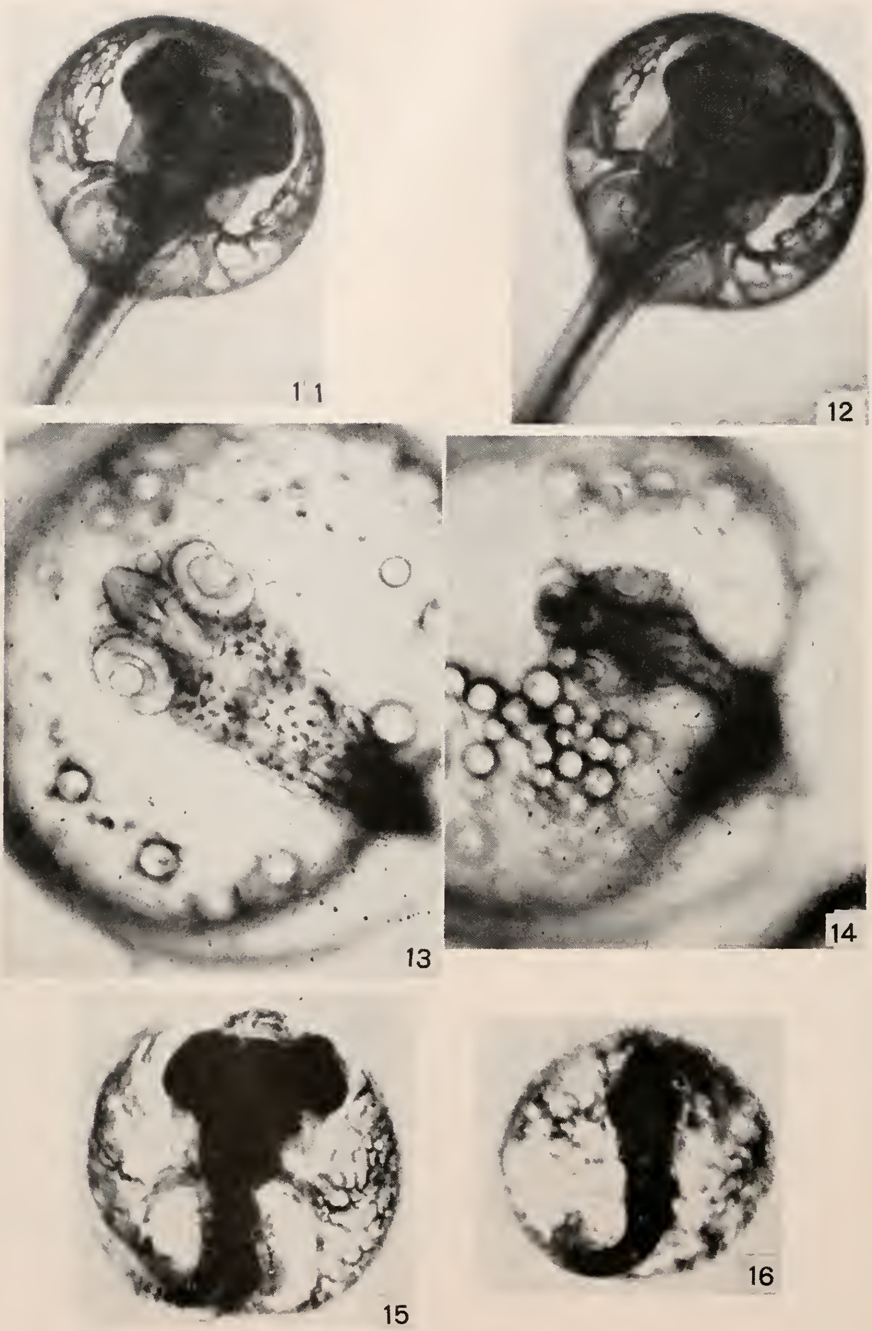
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Plate II

delay in these experiments, the other possible explanations might be considered: (1) Retention of the second polar body; (2) omission of polar divisions; (3) diploidy of virgin eggs; and (4) progressive regulation during cleavage. Information is not available as to the state of the nucleus of *Fundulus* at the time of insemination, and it is unlikely that 3 out of 42 eggs would be diploid before fertilization. Parthenogenetic development in *Fundulus* is most likely to occur from progressive regulation in eggs fertilized by sperm whose genetic complement has been destroyed by 100,000 r or more.

Many of the embryos in these experiments were able to survive for several days, even with poorly developed central nervous system and circulation. The heart was seen pulsating in some embryos even after seven days' development, and some showed muscular movements of the body and tail. It has been found that in haploid amphibian cells the utilization of yolk is much slower than in the controls (Porter, 1939). This might also be correlated with the retardation of carbohydrate metabolism. The amount of oxygen required by haploid cells is not as great as that of diploid cells, enabling them to survive for an extended period without a circulatory supply of oxygen.

A cytological study of control and experimental embryos was made in order to determine whether the parthenogenetic individuals were haploid or diploid, and whether any of their organs reflected these variables. The cells of the gut and kidney were most suitable for counting nucleoli, and the eye for organ development.

The cells of the control embryos in every case possessed paired nucleoli while the majority of the parthenogenetic embryos, from sperm exposed to 50,000 r or more, had single nucleoli. The exception was the embryo from 50,000 r sperm shown in Figure 12, which had two nucleoli per cell. This embryo could not be distinguished from the parallel control, with regard to organ differentiation and development, so that it is presumed to be a recovered-diploid embryo. The chromosomes of this form are almost impossible to count, they are so small and numerous. Many of the embryos from sperm exposed to less than 50,000 r were also probably diploid, possessing two nucleoli in each cell. Thus, such cytological study as was possible corroborated the gross findings of the "Hertwig Effect" even with the fish embryos.

It is of interest that mature sperm of *Fundulus* can tolerate exposures of 150,000 r without impairing their motility or their ability to activate normal eggs.

FIGURE 11. Control embryo at 7 days' development.

FIGURE 12. One of two embryos developing from eggs fertilized with sperm which had been exposed to 50,000 r, shown at 7 days and to be compared with that in Figure 11. Probably diploid.

FIGURE 13. Control embryo at three days of development.

FIGURE 14. An embryo developing from an egg fertilized with sperm which had been exposed to 100,000 r, now seen at three days of age, to be compared with control in Figure 13. Note malformation of brain vesicles, general retardation including the eyes.

FIGURES 15–16. The same embryos (Figures 13, 14) seen at 7 days of development. The experimental (from irradiated sperm) embryo in Figure 16 now possessing circulation. Note poorly developed nervous system, myotomes, and tail, indicating general retardation in development.

The eggs of *Fundulus* are irrevocably damaged so that they cannot develop beyond the stage #15 if they are exposed to as little as 4000 r x-rays.

SUMMARY AND CONCLUSIONS

1. Mature sperm of *Fundulus heteroclitus* were exposed to ionizing radiations from Cs.-137 at the rate of 5000 r/min., for doses ranging from 5000 r to 150,000 r, and were then used to fertilize normal eggs of the same species. Control eggs from the same batch were inseminated with unirradiated sperm from the same source.

2. While variations in normal fertilizability of the control eggs do occur, associated with the season and breeding activity, in every case some eggs were fertilized and developed following insemination with sperm which had been exposed to every dose level. This was not mere activation since cleavages followed.

3. The presence of irradiated (sperm) chromatin had no effect on the time or nature of the early cleavages. The initial adverse effects were noted at the time of gastrulation.

4. Exposures of sperm to 500 r had no apparent effect on the development of eggs, while exposures to 5000 r caused high mortality and morbidity, and above 5000 r (to the sperm) the effect of ionizing radiations appeared to decrease so that a greater percentage of near-normal embryos resulted from cleaving eggs.

5. The fact that a few specimens from 50,000 r or more sperm could not be readily distinguished from the controls suggests that complete recovery of diploidy may sometimes occur after activation. This was substantiated by cytological examination.

6. While parthenogenesis does not, or need not, occur naturally for this species, the fact that it can occur is of biological significance, suggesting that all vertebrates may possess eggs with such regulatory potentialities.

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UPTAKE AND INTRACELLULAR DIGESTION OF PROTEIN (PEROXIDASE) IN PLANARIANS[1]

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It is thought (Willier *et al.*, 1925; Jennings, 1957; Rosenbaum and Rolon, 1960a) that digestion in aquatic planaria is exclusively intracellular, occurring in the spherules of the phagocytic gastrodermal cells. There is, however, very little information concerning the formation of the spherules, the rate of digestion of their contents, and their ultimate fate. Data based on the rate of disappearance of alkaline phosphatase from the spherules in planarians which had been fed raw earthworms (Osborne, 1955) were inconclusive, because of the impossibility of distinguishing exogenous from endogenous enzyme. Nor was it possible in these studies to determine whether the uptake of nutrients occurs exclusively by the phagocytic action of the gastrodermal cells. A new approach to these problems was suggested by the experiments of Straus (1959) on the intracellular disposition of parenterally administered horseradish peroxidase in the rat. Peroxidase, which does not occur in most cells of animal organisms, is readily visualized histochemically and can be used as a tracer for exogenous protein.

MATERIALS AND METHODS

Specimens of *Dugesia tigrina* were starved for 10 days before the administration of peroxidase; this period of starvation is adequate to induce immediate feeding when food is offered but is not long enough to cause extensive resorption of the gastrodermal cells, which would delay the phagocytic uptake of food. Experiments were performed on both normal and pharyngectomized worms.

Pharyngeal feeding of peroxidase was carried out in the following manner, which simulates the conditions of normal feeding. The procedure was suggested by our observation that raw kidney is superior to other commonly-used foods for growth-promotion, and by the report of Straus (1959) that the greatest concentration of peroxidase, following parenteral administration in the rat, occurs in the kidney tubule cells. Mice were given an intracardiac injection of peroxidase (10 mg. in 1 ml. saline), and one hour allowed for glomerular filtration and tubular reabsorption of the enzyme (Straus, 1961). The mice were then killed by decapitation and the kidneys removed and frozen. Thin slices of kidney cortex were placed in jars containing starved planarians, and removed after the completion of feeding, which usually required about 30 minutes. At intervals ranging from 30 minutes to 8 days after the completion of feeding, planarians were removed and fixed for 12–24 hours in cold (4° C.) formol-calcium, rinsed for one hour in cold distilled water (4° C.) and embedded in 10% gelatin. The gelatin blocks were mounted on chilled cryostat

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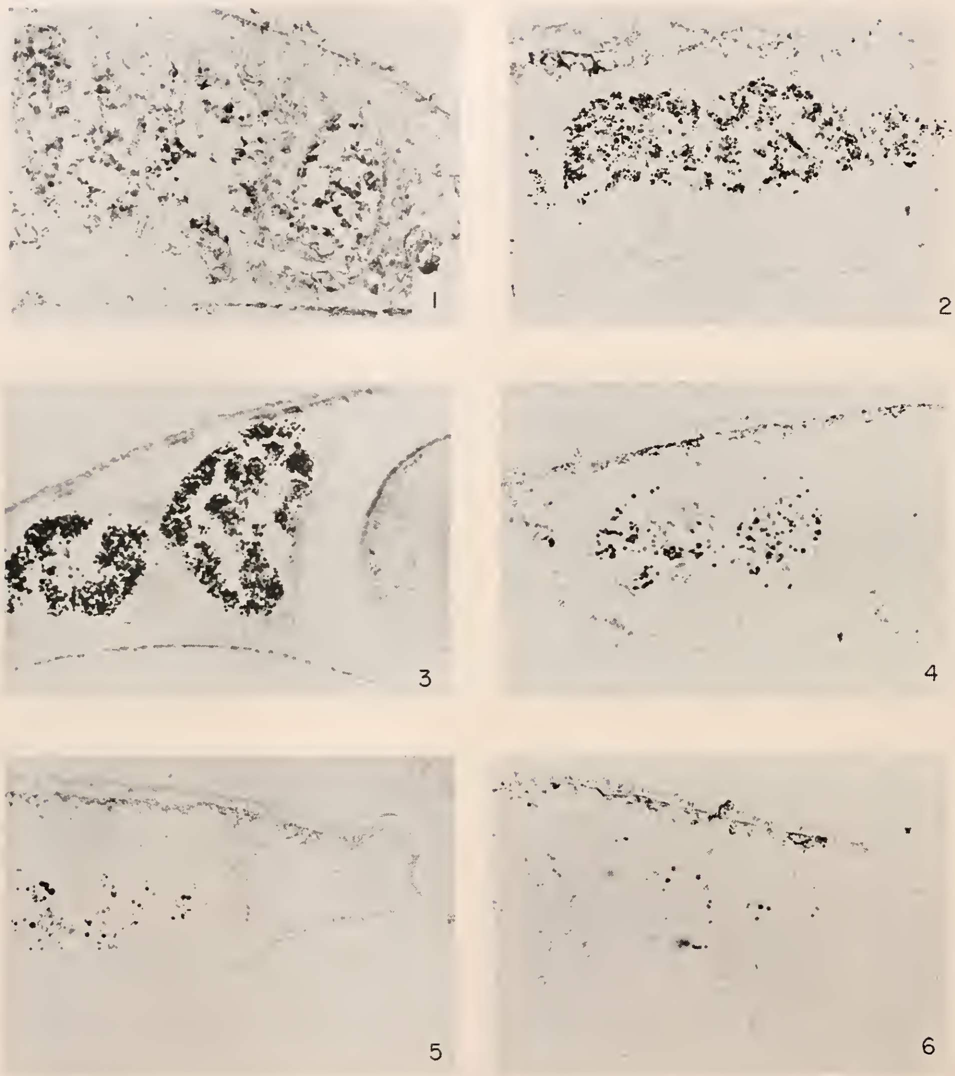


Figures 1–6. Stages in the accumulation and subsequent disappearance of peroxidase reaction product in the phagocytic cells.

Figure 1. Thirty minutes after feeding. Peroxidase activity is localized in diffuse form in the phagocytic cells. 75 ×.

Figure 2. Three hours after feeding. Peroxidase activity is predominantly concentrated in the forming spherules in the phagocytic cells. 75 ×.

Figure 3. One day after feeding. Peroxidase activity is present entirely within spherules of varying sizes. 75 ×.

Figure 4. Three days after feeding. Considerable digestion of the peroxidase has occurred. 75 ×.

Figure 5. Five days after feeding. Many of the spherules no longer show peroxidase activity. 75 ×.

Figure 6. Six days after feeding. Only an occasional spherule shows peroxidase activity. 75 ×.

object holders, frozen by contact with dry ice and cut at 8 μ in a Pearse cryostat. The sections were mounted on chilled slides without adhesive, air-dried for two hours, and the gelatin removed by gentle rinsing in a stream of warm water. The preparations were then incubated at 4° C. for three minutes in the medium recommended by Gomori (1952), as modified by Straus (1959), for the visualization of peroxidase activity. The incubation period was somewhat longer than that used by Straus, but this was necessary for sharp staining of the forming foodballs. Control sections were incubated in a similar manner with the omission of peroxide from the medium.

Permanent preservation of the blue color of the reaction product was achieved by complete dehydration of the specimens through absolute alcohol, followed by clearing in xylene and mounting in Permount.

In order to determine whether or not significant ingestion of protein can occur by extra-pharyngeal routes, planarians were pharyngectomized or transected at the base of the pharynx (which was removed) and the two halves separated from one another. One day was allowed for healing, since McWhinnie and Gleason (1957) have demonstrated that after this interval the cut surface of a transected planarian is covered with epidermis continuous with that of the rest of the organism. Specimens were then placed in solutions of horseradish peroxidase in filtered pond water, in concentrations of 10, 50 or 100 mg. per ml., for periods of 15 minutes to 6 hours. All worms exposed to peroxidase concentrations of 50 mg. per ml. for 6 hours and of 100 mg. per ml. for three hours died, but all survived exposure to 10 mg. peroxidase per ml. for 6 hours. The cause of death after prolonged exposure to higher concentrations of the enzyme is unknown. Control specimens were killed by fixation in cold formol-calcium before being placed in peroxidase-containing media.

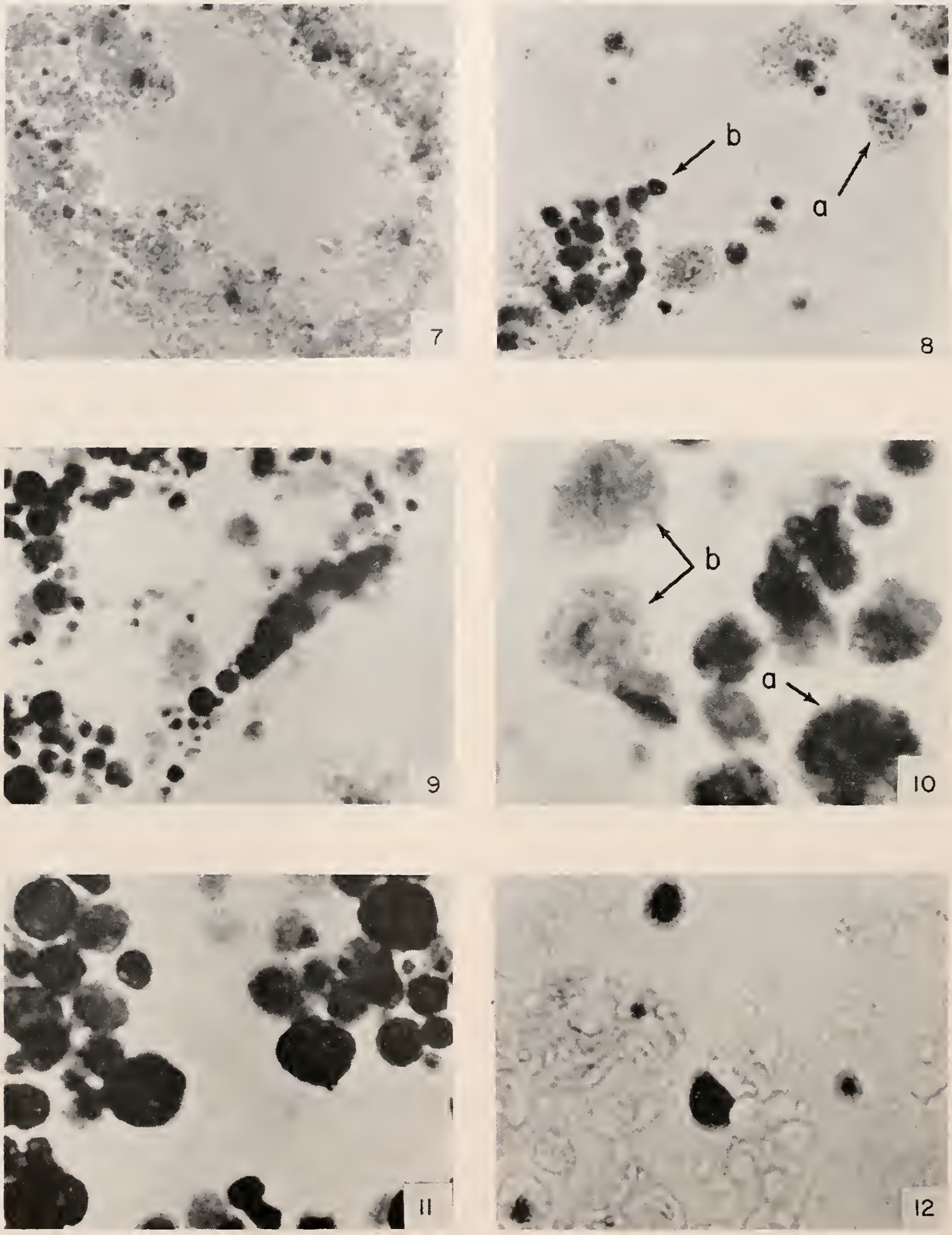
At the end of the exposure periods, the specimens were removed from the peroxidase solution, rinsed in filtered pond water for 5 minutes, and then chilled for a few minutes (4° C.) to produce a non-motile, slightly contracted condition. Cold formol-calcium was poured over each specimen individually, with care to ensure that each was fixed in a smooth, distended state. Fixation and subsequent treatment of these specimens were the same as for the worms fed mouse kidney.

Results

Pharyngeal feeding

In planarians killed 30 minutes after the cessation of feeding there was a diffuse coloration of most of the phagocytic cells with the blue peroxidase reaction product (Fig. 1). Two to three hours following cessation of feeding, the reaction product formed discrete "droplets" within the phagocytic cells of the gut (Figs. 8, 9). Distinct spherules were present in specimens killed one day after feeding (Fig. 3), and gradual disappearance of enzyme activity occurred during subsequent days (Figs. 4–6). No activity was demonstrable on the eighth day following feeding.

Details of the formation of intracellular protein spherules are shown in Figures 7–12. One hour after feeding, the peroxidase reaction product appeared in the form of small granules ranging in diameter from about 0.2 μ up to 2.0 μ (Fig. 7). They were present exclusively within the phagocytic cells; the larger granules appeared to result from the fusion of many smaller ones, since the number and the size of the granules within a cell varied inversely. They increased in size



Figures 7–12. Stages in the formation of peroxidase-containing spherules.

Figure 7. One hour after feeding. Peroxidase reaction product appears as small granules ranging in size from the limit of visibility up to approximately 2 μ in diameter. 600 ×.

Figure 8. Two hours after feeding. Small granules are still present (arrow a), but the largest now approach the dimensions of spherules (arrow b). 600 ×.

Figure 9. Three hours after feeding. Note the phagocytic cell, cut longitudinally, containing numerous spherules and several smaller granules. 600 ×.

progressively (Figs. 8, 9), reaching a maximum diameter of 15–20 μ by the end of one day after feeding (Fig. 11). The classical intracellular "spherules" were clearly seen by the end of the third hour after feeding (Fig. 9). The onset of protein digestion could not be detected by the peroxidase method, but it was well-advanced by the second day after feeding, as indicated by the decrease in the numbers of spherules which gave an enzyme reaction. By the fifth day after feeding, the phagocytic cells were still filled with spherules, but only an occasional spherule contained active enzyme (Fig. 12). Three weeks after feeding, the phagocytic cells contained large numbers of refractile droplets similar to those previously (Osborne, 1955) shown by Nile blue and Sudan III staining to be fat. This suggests a conversion of protein to fat, as has been reported by Willier *et al.* (1925).

Extra-pharyngeal absorption of peroxidase.

When pharyngectomized planarians were exposed to media containing various concentrations of peroxidase, a definite penetration of the enzyme through the intact epidermis could be demonstrated (Figs. 13 and 14), and rows of peroxidase-positive granules were seen extending into the interior. Most of the penetration occurred through the ventral surface, but there was definite evidence of absorption through the dorsal surface as well. In Figure 13, penetration of peroxidase seems to be taking place via the canals left by the extrusion of rhabdites. A few phagocytic cells were filled with peroxidase-positive material within 30 minutes after the beginning of exposure (Fig. 15), and the apparent fusion of smaller into larger intracellular granules is illustrated in Figure 16. After 3 hours' exposure to peroxidase, the typical localization of peroxidase-positive material in the phagocytic cells of the gut was clearly demonstrable (Figs. 17 and 18), although the concentration of enzyme reaction product was very much less than in the case of intact planarians fed peroxidase-containing food.

Specimens killed by formol-calcium fixation prior to exposure showed minimal uptake of peroxidase and absence of intracellular localization.

DISCUSSION

Studies on intracellular digestion in lower organisms are of interest both in their own right and for the light they may shed on similar processes in higher animals. Planarians are particularly well-suited for studies of this type because the phagocytic cells of the gastrodermis are readily accessible to exogenous materials, they respond to the uptake of these materials by the production of a variety of hydrolytic enzymes, and digestion is entirely intracelluar. While changes in enzyme activity associated with intracellular digestion are readily visualized by standard histochemical methods, changes in the materials undergoing digestion have been more difficult to demonstrate. The experiments of Straus

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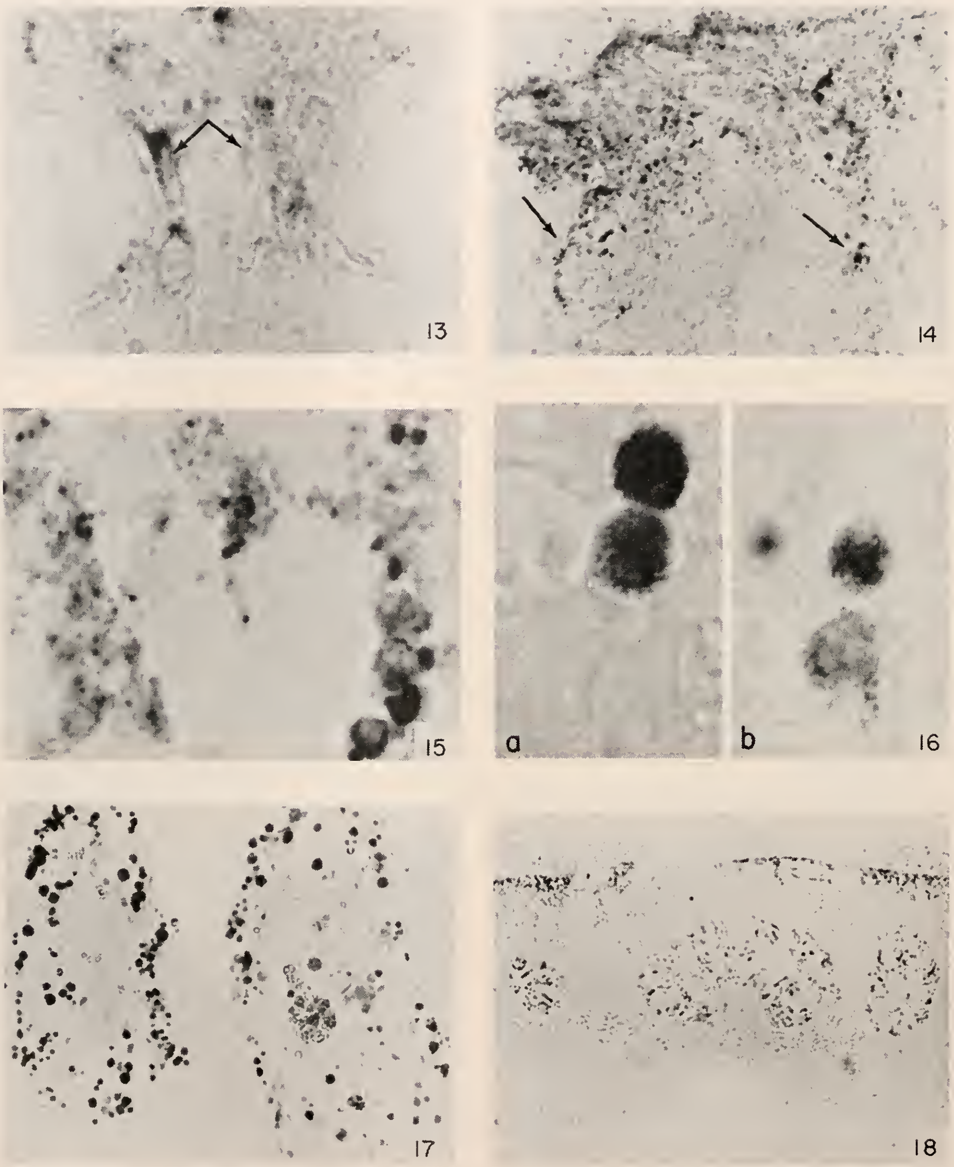
FIGURE 10. Three hours after feeding. Note the phagocytic cell, cut in cross-section (arrow a), containing a number of granules which appear to be coalescing, and other phagocytic cells (arrow b) which still contain many small granules. 1350 ×.

FIGURE 11. One day after feeding. Nearly all the peroxidase reaction product is present in well-formed spherules of varying sizes. 600 ×.

FIGURE 12. Five days after feeding. Large numbers of spherules are now devoid of peroxidase activity. 600 ×.



FIGURES 13–18. Transepidermal uptake of peroxidase in pharyngectomized planarians exposed to solutions of peroxidase in pond water.

FIGURE 13. Exposure time 15 minutes, peroxidase concentration 100 mg. per ml. Arrows point to concentration of peroxidase reaction product in canals from which rhabdites had been extruded. 1000 ×.

FIGURE 14. Exposure time 30 minutes, peroxidase concentration 50 mg. per ml. Arrows point to rows of peroxidase-positive granules extending from the surface toward the interior. 1000 ×.

FIGURE 15. Exposure time 30 minutes, peroxidase concentration 10 mg. per ml. Different stages in the formation of spherules are shown. 600 ×.

(1959) have shown that horseradish peroxidase is readily taken up by the cells of various tissues of the rat, and that its ultimate disposition can be followed by histochemical methods. This provides a convenient procedure which should be applicable to studies on the uptake and digestion of exogenous protein by the phagocytic cells of lower forms; the major uncertainty in the interpretation of the results concerns the degree of degradation of the enzyme molecule necessary for the abolition of its enzyme properties. It is interesting to note that the rate of disappearance of exogenous peroxidase activity from the phagocytic cells in the present study is in approximate agreement with the rate of disappearance of food spherules reported by Willier *et al.* (1925), based on non-specific histological staining methods.

The formation of intracellular food spherules appears to involve the progressive fusion of large numbers of very small granules. The smooth, circular profile of spherules of varying sizes suggests the presence of a limiting membrane of the type commonly associated with food vacuoles, a supposition which is supported by the presence of spherules devoid of peroxidase activity some days after the ingestion of peroxidase-containing food (Fig. 12). The mechanism of formation of these spherules is obscure and might profitably be studied by electron microscopy.

The transepidermal uptake of peroxidase in pharyngectomized planarians suggests a possible route by which nutrients are absorbed from the medium by planarians undergoing regeneration after transection or binary fission. Further work will be required before the quantitative importance of transepidermal absorption can be assessed.

While this material was being prepared for publication, our attention was drawn to the paper of Rosenbaum and Rolon (1960b), thus far available only in abstract form, in which the absorption by planarians of peroxidase dissolved in the medium was reported. Our results are in general agreement with theirs, although the procedure differed somewhat in the two experiments; the major difference in results is the much greater toxicity of peroxidase in our experience, the basis of which is to be investigated.

SUMMARY

1. Stages in the ingestion of protein, and the formation and ultimate disappearance of spherules in the phagocytic cells of *Dugesia tigrina* were visualized histochemically by the peroxidase technique.

2. The formation of spherules involved the coalescence of numerous small peroxidase-positive granules. Typical spherules were present three hours after feeding kidney from a mouse previously injected with peroxidase, and maximal size of the spherules was achieved by the end of 24 hours.

FIGURE 16. Exposure time one hour, peroxidase concentration 100 mg. per ml. Earlier (b) and later (a) stages in the aggregation of peroxidase-positive granules in the phagocytic cells. Other phagocytic cells, as yet devoid of enzyme activity, are also present. 1350 ×.

FIGURE 17. Exposure time three hours, peroxidase concentration 50 mg. per ml. Typical spherules are present in the phagocytic cells. 300 ×.

FIGURE 18. Exposure time three hours, peroxidase concentration 50 mg. per ml. The enzyme activity is localized in the phagocytic cells but in much smaller amounts than after pharyngeal ingestion of peroxidase-containing mouse liver (*cf.* Fig. 2). 75 ×.

3. Peroxidase-positive material was confined to the phagocytic cells and disappeared gradually until none remained 8 days following feeding.

4. Pharyngectomized planarians exposed to a medium containing peroxidase in solution absorbed the protein through the epidermis, and formed typical spherules in the phagocytic cells. It is suggested that this may indicate a role of transepidermal absorption of nutrients in regenerating planarians.

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FILTER-FEEDING PATTERN AND LOCAL DISTRIBUTION OF THE BRACHIOPOD, DISCINISCA STRIGATA

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The recent surge of interest in the functional morphology of living brachiopods has been led mainly by paleontologists, motivated by an intent to increase the reliability of their interpretations of fossil structures. Research has been focused on the mechanics of feeding, since the morphology of the filtering organ, the lophophore, and, where present, its supports, is basic to understanding the phylum's systematics. Orton (1914) contributed the first paper in this series and has been followed by Richards (1952), Atkins (1956–1961; see Rudwick, 1962, for complete citations), Chuang (1956), Williams (1956, 1960), Rudwick (1960a, 1960b, 1962), Rowell (1961), and Williams and Wright (1961). These works suggest that, with the possible exception of *Discinisca* (Rowell, 1961), all Recent brachiopods show a fair degree of convergence in the characteristics of their feeding and the fleshy portions of their lophophores. On the other hand, the functioning of "fossil" feeding mechanisms has not been agreed upon. This paper describes the filtering mechanism of the inarticulate brachiopod, *Discinisca strigata* Broderip, which, because of its uniqueness, will permit some features common to all known brachiopod filtering mechanisms to be evaluated.

MATERIAL

Large numbers of *D. strigata* were discovered living in the tidal zone near Puertecitos, Baja California, Mexico (approximately 30° 17′ N.; 114° 40′ W.). Field observations were made from 29 March to 1 April, 1962, principally at a station 2–3 km. north of Puertecitos, and were facilitated by an estimated 20-foot tidal range. Living specimens were successfully transported back to the Scripps Institution of Oceanography where details of the feeding process were worked out.

ECOLOGICAL OBSERVATIONS

At the principal station an extensive sand beach is interrupted at regular intervals by patches of cobbles and small boulders extending down to the low-water mark. *D. strigata* lives under the flatter of these rocks, conspicuously associated with the sponge, *Hymeniacidon adreissiformics* Dickinson, the gastropods, *Acanthina angelica* Oldroyd, *Nerita funiculata* Menke, and *Morula ferruginosa* Reeve, and the bivalves, *Barbatia reeveana* (Orbigny) and *Isognomon chemnitzianus* (Orbigny). Whether suitable habitats in other areas can be recognized by the presence of these species is not yet known. The brachiopods often had settled in small crevices in the rocks, and occurred in microenvironments probably characterized by reduced current scour. The size distribution of the

Puertecitos population (Fig. 1), based on measurements of all specimens obtained from a large (200 × 50 yards) area of the cobble patch, shows no multimodality indicative of either two or more breedings or a number of year classes. The position of the normally shaped curve suggests a single prolonged spawning sometime prior to the date of collection, and that the animals may be annuals.

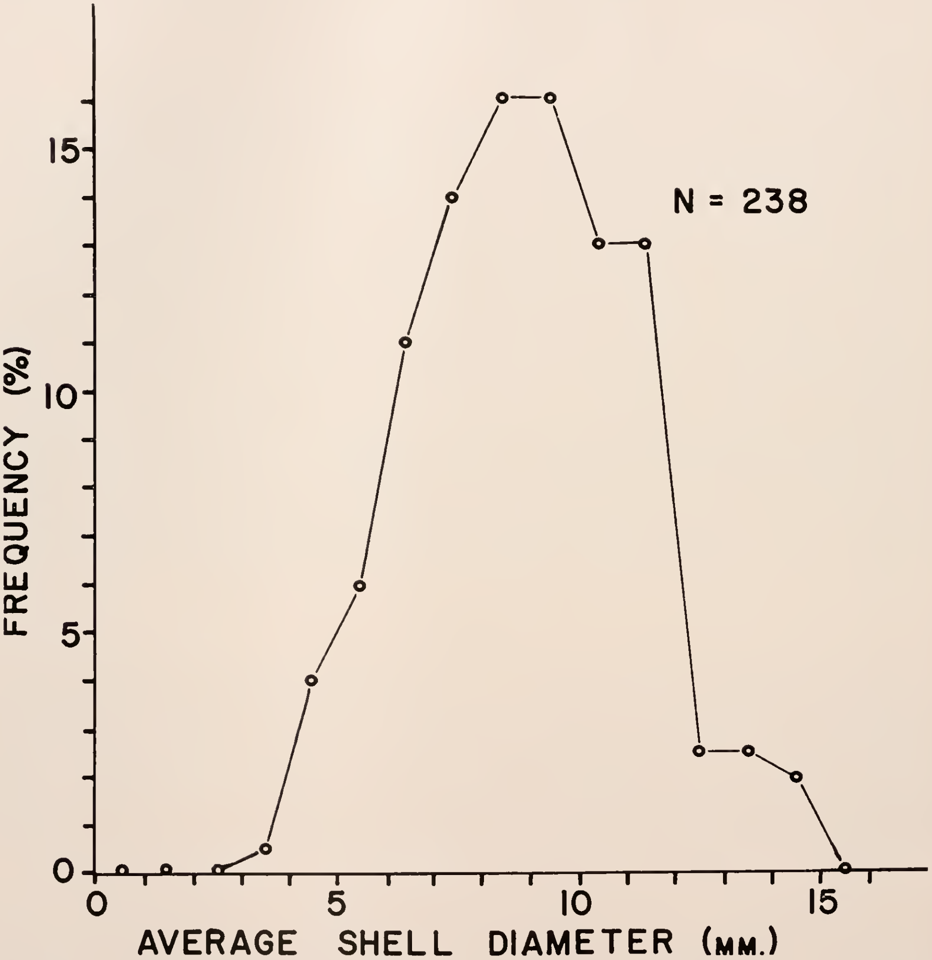

FIGURE 1. Diameter-frequency distribution of *D. strigata*. The diameter of each specimen is composed of an average of its length and width; the use of this measure is necessitated by the extremely variable individual shape.

Like many marine species, *D. strigata* shows signs of zonation. One cobble patch was completely exposed on an extreme low tide, permitting a transect to be made (Table I). Six flattish rocks, each about a foot square on their lower surface, were overturned at 10-foot intervals from the beginning of the cobble patch to the high-water mark, and the number of brachiopods seen recorded for each interval. Brachiopod distribution was curtailed at both seaward and landward extremes, although seemingly acceptable rocks existed throughout the body of the reef. The position of this upper limit was further substantiated by two less complete transects. The lower limit cannot be explained at present, but desiccation appears to influence the upper extension. When adjacent flat rocks, high in the intertidal, were chosen such that one was slightly more elevated than the other, *D. strigata* was always under the wetter of the two rocks. Although G. A. Cooper (in Cloud, 1948) has also taken this species in intertidal water, the type was dredged from 18 fathoms (Broderip, 1833). Zonation at Puertecitos may thus be a local phenomenon, or possibly the type was collected at an atypical depth.

The evidence is substantial that this species, unlike other discinids, never forms clusters of many individuals. From collections at three Baja California locations, San Felipe, Puertecitos, and Bahia de Los Angeles (Courtesy of B. N. Kobayashi) 11, 238, and 2 specimens, respectively, were scanned with a microscope. Although all degrees of incrustation existed, no small brachiopods were observed. In addition, by inference the type specimens from Guatemala were collected singly (Broderip, 1833) as were those obtained by G. A. Cooper (personal communication) near Matzatlan, Mexico. On the other hand, clusters of *D. lamellosa* and *D. laevis* are well-documented (Davidson, 1888), implying the existence of distinctive differences in larval settling behavior within the genus. Blochmann (1908) has suggested that discinid clustering was associated with poor dispersal powers. It seems as probable, however, in light of an increased knowledge of the role of site selection exercised by certain larvae (Wilson, 1958), that the intrageneric variation stems from differences in settling behavior.

TABLE I

Zonation of D. strigata *based on a transect. Numbers indicate the quantity of brachiopods observed under 6 flat rocks in each 10-foot interval. Distances are given in feet from the high-water mark. The vertical excursion is approximately 20 feet.*

Distance		0–160			170		180		190		200		210		220	
Numbers		0		1		0		5		20		25		12		6
Distance	230		240		250		260		270		280		290		300	
Numbers		5		7		11		17		0		3		3		2
Distance	310		320		330		340			350–500						
Numbers		2		1		1		1		0						

FILTER-FEEDING MECHANISM

Hyman (1959) has cited the extensive and varied elaboration of the brachiopod lophophore as characteristic of the phylum. One diverse group of Recent species, drawn from both the Inarticulata and Articulata and encompassing four families, can be described as having a spirolophous lophophore (spirolophes); that is, in the mature individual the lophophore is coiled into two simple arms which, in all known spirolophes except *Discinisca* and the closely related *Discina* (Rowell, personal communication), point dorsally. Each arm (or brachium) bears a double row of ciliated filaments separated by the brachial groove from the lip. The side containing the lip is termed the frontal surface, and lateral cilia on the filaments beat across the length of the filament from the frontal toward the abfrontal surface (Atkins, 1956). Thus, the organism's food is filtered from water currents drawn perpendicularly to the length of the filament in a frontal to abfrontal direction. The interested reader is referred to Rudwick (1962) for a more complete account of the ontogeny and functioning of a variety of brachiopod lophophores.

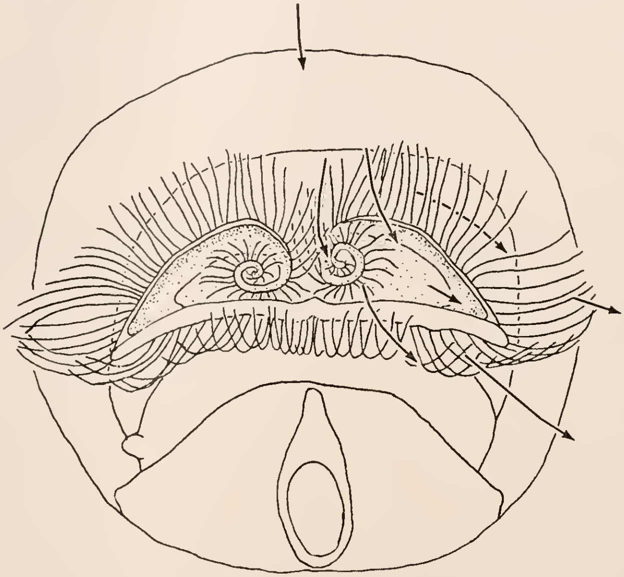

FIGURE 2. View of the expanded lophophore of *D. strigata* after removal of most of the ventral valve. The arrows represent the observed direction of water flow across the lophophore. The setae fringing the shell have been omitted.

The above arrangement of spiral arms, filaments, and ciliary beat specifies the feeding currents of most spirolophes. However, the ventrally-directed *Discinisca* spires have paleontological implications and the situation needs clarification.

The ventral valves of a number of *D. strigata* were partially removed and the lophophores allowed to relax fully, without narcotization. The water currents around the lophophore were then drawn as they appeared when viewed from the ventral aspect (Fig. 2). The apices of the slightly elevated spires are oriented ventrally (pointed toward the observer) and perhaps canted toward the median plane. The proximal whorl of each brachium appears greatly expanded, extending from the median plane almost to the lateral margin of the shell. The remaining more distal whorls comprise an insignificant portion of the spire, and, in fact, give rise to less than 20% of the total filament length. This figure was arrived at by measuring scattered filaments on the proximal and more distal whorls, and then multiplying their mean lengths (1.7 mm. and 0.5 mm., respectively) by their numbers. Presumably, as the animals become larger, the disparity in allocation of filtering surface will continue to increase in favor of the proximal whorl.

In Figure 2 the frontal surface of the anterior filaments of the proximal whorl faces the observer, and in this region water currents pass normal to the plane of commissure (into the plane of the paper). These anteriormost filaments tend to lie against the mantle, forming an exhalant chamber leading to the posterolateral margins, dorsal to the main body of the lophophore. Water filtered by these filaments occupies this dorsal chamber. Similarly, if the ventral shell and mantle were present, those posterior filaments behind the mouth and closest to the observer, in lying against the mantle, would form another analogous exhalant chamber ventral to the lophophore. Some unfiltered water is also drawn into the distal portions of the spire and after being filtered must move dorsally and then laterally to join the main exhalant currents at the posterolateral margin of the valves. This previously filtered water inside each spire cannot mix with the unfiltered water because the filaments on the distal whorls are flexed abfrontally, touching the frontal surface of the next more proximal whorl. Most of the unfiltered water, however, passes along the expanded body of the proximal whorl and is filtered by the long filaments toward its lateral end.

As in most brachiopods, the filaments are incompletely ciliated, a tract being absent from the abfrontal surface. On excised filaments the lateral cilia were usually still, but the frontal ones continually beat toward the filament tips. Their normal beat would be toward the base and adjacent food groove, and this beat reversal can serve as a rejection mechanism.

At least two such rejection mechanisms are functional in the whole organism. The beat of the frontal cilia appears to be frequently reversed, since bands of mucus-bound particles were seen to be carried away from the brachial lip. And heavy particles, once inside the inhalant chamber, are pushed toward the chamber's margins by a coordinated flexing of the filaments, similar to that illustrated for *Lingula unguis* by Chuang (1956). Once at the edge these particles are probably expelled by a gentle clapping shut of the valves, as is known in the inarticulates, *Crania* (Orton, 1914) and *Glottidia* (Paine, unpublished), and some articulates (Rudwick, 1962).

In the normally feeding intact animal the slight gape of the valves is masked by a heavy fringe of long, barbed setae. The gross current pattern consists of a single, median inhalant current and paired, posterolateral exhalant ones. This pattern was never strongly developed, especially the exhalant currents, though in 20 specimens examined little variation was noticed. Not much current distortion is caused by the fringing setae which function, aside from being tactile elements, to catch and hold all but the finest water-drawn particles. In freshly collected specimens these setae were invariable festooned with detritus, particularly around the median inhalant aperture where they also reach their maximum length.

The nature of the currents inside the unopened animal can only be speculated on. Most likely all filaments of the proximal whorl touch the dorsal or ventral mantle surfaces. The tips of the longer, laterally placed filaments also probably intermesh completely in the restricted space of the mantle cavity, and in so doing form a tunnel trending laterally which encloses the main body of unfiltered water. The apices of the spires, enclosed within this tunnel, will be canted toward the median plane and possibly serve to orient the incoming current.

Discussion and Conclusion

A revival of interest in brachiopods, apparently focused on their feeding mechanism, has shown that most species can accurately be characterized by certain generalities. (1) In adult specimens the unfiltered water enters the mantle cavity as paired lateral currents and exits as a single median one, although in small individuals this external current pattern is usually reversed (Atkins, 1956; Rudwick, 1962). (2) The feeding currents are mainly produced by lateral cilia on the lophophoral filaments beating in a frontal to abfrontal direction, and the beat of these is seldom reversed. The report by Atkins (1960) of current reversal in the Megathyridae represents a momentary phenomenon; the usual beat is similar to that in other brachiopods and Atkins suggests (p. 471) that the reversal is elicited ". . . when strong cleansing action is called for." (3) And in spirolophous brachiopods the apices of the spires point dorsally.

Rudwick (1960a) has added further considerations. Filtered and unfiltered water may be kept separate by the arrangement of the lophophoral filaments. If this is achieved the animal possesses an "efficient" filtration system, if not, an "inefficient" one. All Recent brachiopods appear to be "efficient." Second, when unfiltered water occupies the center of the spire, the current system can be called inhalant, a condition characterizing most living spirolophes. An alternative inferred for *Discinisca* on topological grounds by Rowell (1961), and potentially equally efficient, is an exhalant system characterized by previously filtered water in the spire's center. Finally, Rudwick (1960a) has shown that all spirolophes can fall into either of two mutually exclusive categories. When the left brachium is viewed from its base toward its apex, it will coil either clockwise or counterclockwise. Among Recent species the latter group includes most spirolophes, the former only *Discinisca* (and presumably *Discina* as well). Both categories are well represented in fossil forms. Because the frontal surfaces of the brachia always face the mouth in early lophophoral stages, the counterclockwise group eventually develops an inhalant system and the clockwise group an exhalant one.

Rudwick's (1960a) contention that extinct spirolophes had only inhalant or exhalant systems of the construction presently extant has been challenged by Williams (1960) and Williams and Wright (1961). Much of what has been learned about *Discinisca* cannot help to resolve the central issues of this debate, which involve possible functions of structures only present in articulate brachiopods. However, because the left brachium of the *Discinisca* spire does coil clockwise, analogies drawn with extinct spire-bearers also characterized by clockwise coiling of their left brachium become more reasonable. And, through comparison with other spirolophes, those characteristics of the brachiopod feeding mechanism which are independent of lophophore orientation can be specified.

Perhaps the most germane observations are that the basic construction of the brachium has not been altered, and that the lateral cilia continue to beat in a frontal to abfrontal direction. However, the filaments of *D. strigata* are flexed abfrontally rather than frontally as in the inarticulates, *Lingula* (Chuang, 1956) and *Crania* (Atkins, in Rudwick, 1960a). The result of this alteration in flexure is that, given a ventrally-oriented spire, the feeding mechanism retains its efficiency. As Rowell (1961) has shown, if the filaments were flexed frontally, the inhalant and exhalant chambers could not be effectively isolated and the system would be inefficient. The filaments, flexed as they are in *D. strigata,* form a system of inhalant and exhalant spaces which prevent previously filtered water from being recycled before it has been pumped from the animal. With the retention of the usual direction of ciliary beat and the maintenance of an efficient system, the gross current pattern can only be as it is.

D. strigata, however, shows a mixture of inhalant and exhalant systems. The water in the central portions of the spire already has been filtered, and on this basis this species must be considered at least partially exhalant. However, only 20% of the water is processed in this manner, the remainder probably passing along the tunnels formed by the lateral extension of the proximal whorl. The water here, in what would normally be the inside of the spire, is unfiltered and thus fits Rudwick's (1960a) description of inhalant systems. Blochmann's (1900; plate 8, Fig. 10) illustration of *D. lamellosa* suggests that a similar extension of the proximal whorl is found in another *Discinisca* species, and thus that a combination of inhalant and exhalant systems may typify the genus. This finding does not alter the logic of Rowell's (1961) conjecture based on a *Discinisca* lophophore in which all the whorls are concentric and similarly shaped, and where the filaments of the spire touch the next more proximal whorl. It should be emphasized that if the terms inhalant and exhalant current systems are simply descriptive, then *D. strigata* belongs to the exhalant group because only filtered water lies within the central axis of the spire. However, if total lophophore functioning is also considered, the dual interpretation of *D. strigata* is reasonable.

These results suggest that problems associated with gross current pattern, orientation of the spire, and the presence of inhalant or exhalant filtering systems can be minimized in typically constructed species, both extant and fossil. Reconstruction of the lophophores of fossil spirolophes can probably be satisfactorily based on the consistent properties of efficient filtering and unidirectional ciliary beat in a frontal to abfrontal direction.

This work was done during the tenure of a Sverdrup Postdoctoral Fellowship at the Scripps Institution of Oceanography. The author wishes to thank the following specialists for specific identifications: Dr. G. A. Cooper (brachiopod), Dr. W. D. Hartman (sponge), and Dr. R. Stohler (Mollusca). The paper has benefited from the suggestions of and critical reading by Dr. A. J. Rowell. Special recognition is due Mr. E. P. Chace, of the San Diego Museum of Natural History, for suggesting the locality where the *Discinisca* were found.

SUMMARY

1. Ecological observations on the brachiopod, *Discinisca strigata,* suggest that this species is zoned in shallow water in the northern Gulf of California. It occurs singly rather than in great clumps, and on the basis of a size-frequency distribution appears to be an annual.

2. Examination of the filter-feeding apparatus and its operation shows, despite an exceptional orientation of the lophophore, that there are a number of points in common with other brachiopods. The lateral lophophoral cilia beat in a frontal to abfrontal direction, and the current system through the animal is efficient.

3. The position of the inhalant aperture, relative direction of coiling of the left brachium, and orientation of the spire, although differing from those in other adult spirolophes, do not diminish the efficiency of operation. *D. strigata,* though showing a mixture of inhalant (80%) and exhalant (20%) filtering systems, is able to maintain its filtering efficiency, due to the disposition of filaments within the organism.

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THE HEMOGLOBIN OF THE BIVALVED MOLLUSC, PHACOIDES PECTINATUS GMELIN

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Hemoglobin has been reported from only a few species of bivalved molluscs; it occurs in cells in the blood of *Poromya granulata, Solen legumen, Tellina planata, Capsa fragilis, Cardita aculeata, Arca tetragona, Arca noae, Pectunculus glycimeris, Astarte fusca* (?) Griesbach, 1891), *Cardita sulcata* (Paladino, 1909), *Anadara inflata* (Kawamoto, 1928), *Arca pexata* (Svedberg and Hedenius, 1934) and *Arca subcrenata* (Kobayashi, 1935). In *Tivela stultorum* hemoglobin occurs in the brain (*sic*), mantle, gills, foot and adductor muscle (Fox, 1953).

Studies of absorption spectra have been carried out on the hemoglobin of *Arca subcrenata* by Kobayashi (1935), and Svedberg and Hedenius (1934) have run ultracentrifuge sedimentation studies on the pigment of *Arca pexata;* biochemically, however, the most extensively studied lamellibranch hemoglobin is that of *Anadara inflata*.

The study of *Anadara inflata* hemoglobin began with the work of Kawamoto (1928), who reported the oxygen dissociation curve of the pigment. Sato (1931) and Kobayashi (1935) established the absorption spectra of the pigment and its derivatives. Work on the hemoglobin of *Anadara inflata* culminated with the efforts of Yagi *et al.* (1955a, 1955b), who purified the hemoglobin, determined its molecular weight from sedimentation and diffusion studies, its electrophoretic mobility, iron content, nitrogen content and N- and C-terminal amino acids; in addition, molar extinction coefficients were also reported.

The present communication deals with the hemoglobin of the lucinid pelecypod, *Phacoides pectinatus* Gmelin, which the author chanced on in Puerto Rico. This animal lives deep (down to 18 inches) in the mud of mangrove swamps, and when it is opened exhibits dark purplish ctenidia with an appearance reminiscent of ripe muscatel grapes. The bloom on the surfaces of the ctenidia is due to a superficial layer of pigment-free cells; however, when the ctenidium is torn, it exhibits a bright red interior suggestive of hemoglobin. For illustration of the appearance of the gills see Figure 1. Although the clam is commonly eaten, at least in the neighborhood of La Parguera, P. R., and must therefore be somewhat well known, the author can find no mention of its hemoglobin in the literature.

A four-sided study of the red pigment of the ctenidia of *Phacoides pectinatus* has been undertaken. First, evidence for the identity of the pigment with hemoglobin has been obtained from studies of absorption spectra; second, the oxygen-combining properties of the pigment have been studied; third, the behavior of the pigment in the ultracentrifuge has been examined with a view to gaining some idea of the size of the molecule; and fourth, the histology of the pigment has been studied.

MATERIALS AND METHODS

General

Phacoides pectinatus Gmelin was collected in the neighborhood of Point Pitahaya near La Parguera, Puerto Rico. They were stored at the Marine Station of the University of Puerto Rico on Magueyes Island, La Parguera, in running sea water at a temperature ranging between 26 and 28° C.; they were then taken back to the Biological Laboratories, Harvard University, where they were stored in aerated sea water at room temperature.

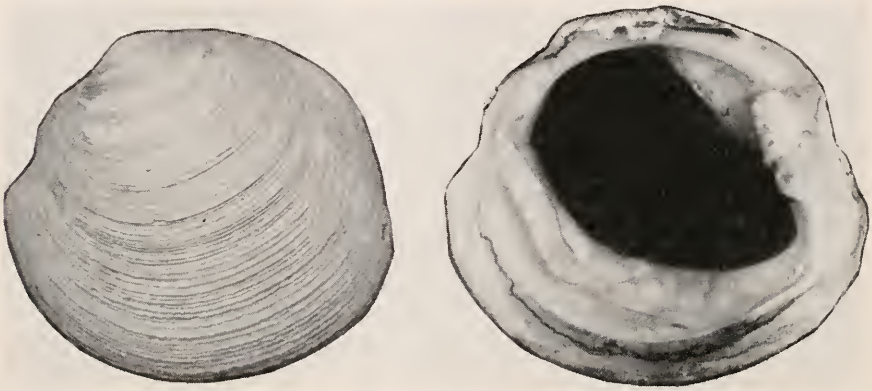

FIGURE 1. *Phacoides pectinatus* Gmelin. Length 5 cm. Dorsal side uppermost. The large dark mass shown by the dissected animal is the right ctenidium; the dark color is due to hemoglobin. Note the colorless edge of the demibranch, which is an indication of the hemoglobin-free, ciliated cells of the exterior of the gill. Members of the family Lucinidae have only the outer demibranch (Purchon, 1939).

Absorption spectra

Centrifuged homogenates of ctenidia, in phosphate buffers ranging in pH from 6.8 to 7.5 and molarity from 0.067 to 0.2 *M*, were examined spectroscopically with a Beckman Model DB recording spectrophotometer. Deoxygenation was brought about either by washing the preparation repeatedly with nitrogen or by adding a small amount of sodium dithionite. Carboxyhemoglobin was made by bubbling carbon monoxide through the dithionite-treated preparation or through the untreated preparation of oxyhemoglobin.

Oxygen dissociation curve

Samples of hemoglobin were prepared by homogenization of dissected ctenidia in a glass homogenizer with pH 7.4, 0.2 *M* phosphate buffer at 3° C. The homogenate was centrifuged at 8,000 × *g* for ten minutes to remove the coarser cellular debris, and the resulting supernatant was further centrifuged at 100,000 × *g* in the ultracentrifuge for 30 minutes.

After ultracentrifugation, the clarified preparation was transferred to tonometers of known volume, and the oxygen dissociation curve of the pigment determined by the method of Riggs (1951), slightly modified. In Riggs' method the solution of hemoglobin in a tonometer, of which a cuvette for spectrophotometry forms an integral part, is freed of all oxygen by repeated washing with nitrogen; measured amounts of air are then introduced by way of syringes into the tonometer, and after each addition the absorption spectrum of the hemoglobin solution is measured. The oxygen tensions resulting from each increment of added air must be computed with regard being taken for the vapor pressure of water, oxygen dissolved in the water of the hemoglobin solution and oxygen combined with the hemoglobin itself; the degree of saturation of the hemoglobin with oxygen is computed from measurements of the absorption spectra at 560 mμ. The volume of the tonometers employed was about 430 ml. and the amount of hemoglobin solution 8 ml.; the amount of hemoglobin in solution was estimated from measurements of its optical density at the α peak and an assumed millimolar extinction coefficient of 15. The absorption spectra were measured with Beckman Model DB or Cary Model 11MS recording spectrophotometers. At the end of each run a check against leakage was automatically provided, since the actual pressure within a tonometer could be compared with the pressure computed from measurements of the initial pressure and the varying increments of added gas; the results were deemed acceptable if the pressures obtained by the two methods agreed within 1 mm. Hg.

Sedimentation constant

A sample of centrifuged homogenate of ctenidia in pH 7.4, 0.2 *M* phosphate buffer was placed in the cell of a Spinco Model E analytical ultracentrifuge and the sedimentation constant determined at 59,780 rpm with a Wratten No. 2412 filter (red) in the optical system.

The sedimentation constant s of a protein is the velocity of sedimentation of the protein molecule in a field of centrifugal force of unit strength under the particular conditions of medium and temperature at which the determination is performed. A correction factor can be estimated from tables given by Svedberg and Pedersen (1940), whereby the experimental value of s may be corrected for viscosity and density effects to a base of 20° C. and water; the value for the sedimentation constant under these conditions is given the symbol $s_{20,w}$.

As a protein sediments it leaves a volume of solution clear of that particular protein directly behind it so that a boundary is formed. The concentration gradient represented by the moving boundary is converted into a peak by an optical system and the position of this peak is photographed at intervals of time; this allows the rate of sedimentation of the protein to be followed. Now there may be several proteins in the sample, and if one of them is colored, a change of density of film exposure will be associated with the colored protein's peak, since along with the peak the contents of the sedimentation cell are also photographed. Thus, in Figure 3 if it is assumed that the only colored protein present is hemoglobin, then this will be the protein associated with the slow peak and as such it has been taken.

Histology

The ctenidial tissue of *Phacoides pectinatus* was treated in two ways. The first approach was the examination of unstained cryostat sections, while the second

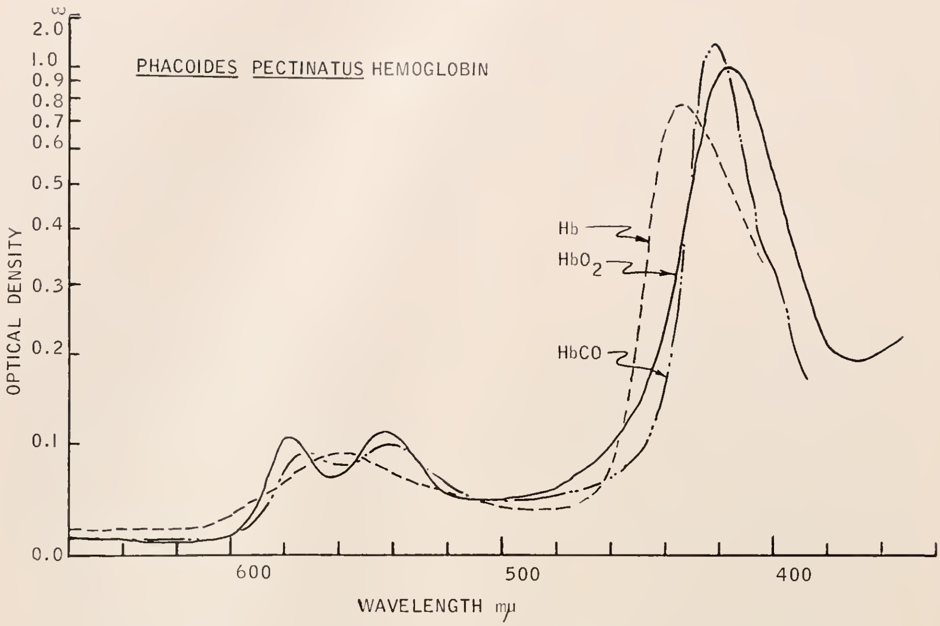

FIGURE 2. Absorption spectra of *Phacoides pectinatus* hemoglobin and derivatives.

included the fixation of the tissue in 10% acrolein in xylene at 0° C., embedding in wax and staining for occult iron by the methods of Glick and Hutchison as described in Humason (1962), somewhat modified.

The most satisfactory method for the iron stain proved to be the following: (1) Deceration and hydration of the tissue to water; (2) two minutes in 30% H_2O_2 alkalized with one drop concentrated NH_3 solution per 100 ml.; (3) thorough rinsing with several changes of distilled water for 5 minutes; (4) 15 minutes at 56° C. in a freshly made, filtered and heated solution of acidified potassium ferrocyanide, made by mixing 25 ml. each of solutions containing 2 g. potassium ferrocyanide to 50 ml. water and 2 ml. concentrated HCl to 50 ml. water; (5) thorough rinsing with several changes of distilled water for 5 minutes; (6) counterstaining 4 minutes in 1% carminic acid; (7) 5 minutes' differentiation in 4% potassium aluminum sulfate; (8) thorough rinsing in several changes of distilled water for 5 minutes; (9) dehydration, clearing and mounting. The iron-containing material is stained a bright green and the nuclei red; the remainder of the tissues stain various shades of pink.

RESULTS

Absorption spectra

The results of the measurement of the absorption spectra of the hemoglobin of *Phacoides pectinatus* are shown in Figure 2. In the following description the numbers in parentheses represent the range of the observations in mμ and the number of observations, respectively. The *Phacoides pectinatus* preparation in the oxygenated state shows strong α, β and Soret bands with peaks at 578.7 (1,6), 543.3 (2,6) and 416.3 mμ (1,3), respectively; upon deoxygenation the α and β bands disappear, to be replaced by a single band with a peak at 558.1 mμ (2,6), while the Soret peak shifts to 433.5 mμ (1,2); treatment of the preparation with carbon monoxide causes reappearance of the α and β bands, but shifted towards the violet compared with oxyhemoglobin, and their peaks lie at 572 (0,2) and 541 mμ (0,2), respectively; the Soret band of the carbon monoxide-treated prepa-

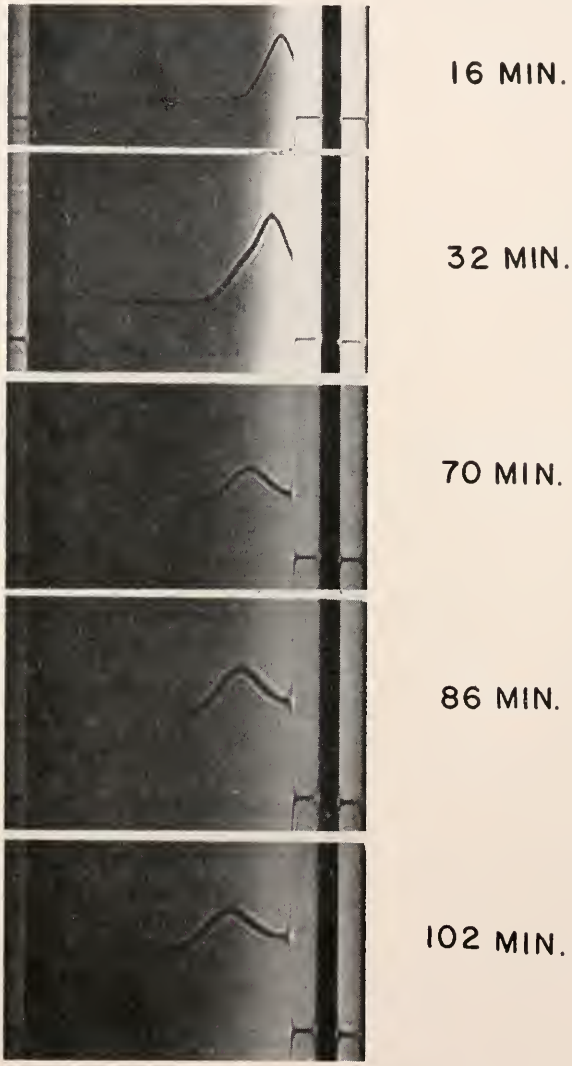

FIGURE 3. Ultracentrifuge run. The hemoglobin is sedimenting from right to left. Note how the larger schlieren peak is associated with the change in optical density in the cell, indicating that it is probably the hemoglobin peak. The 16- and 32-minute photographs were taken on a different plate from those taken during the interval 70 to 102 minutes.

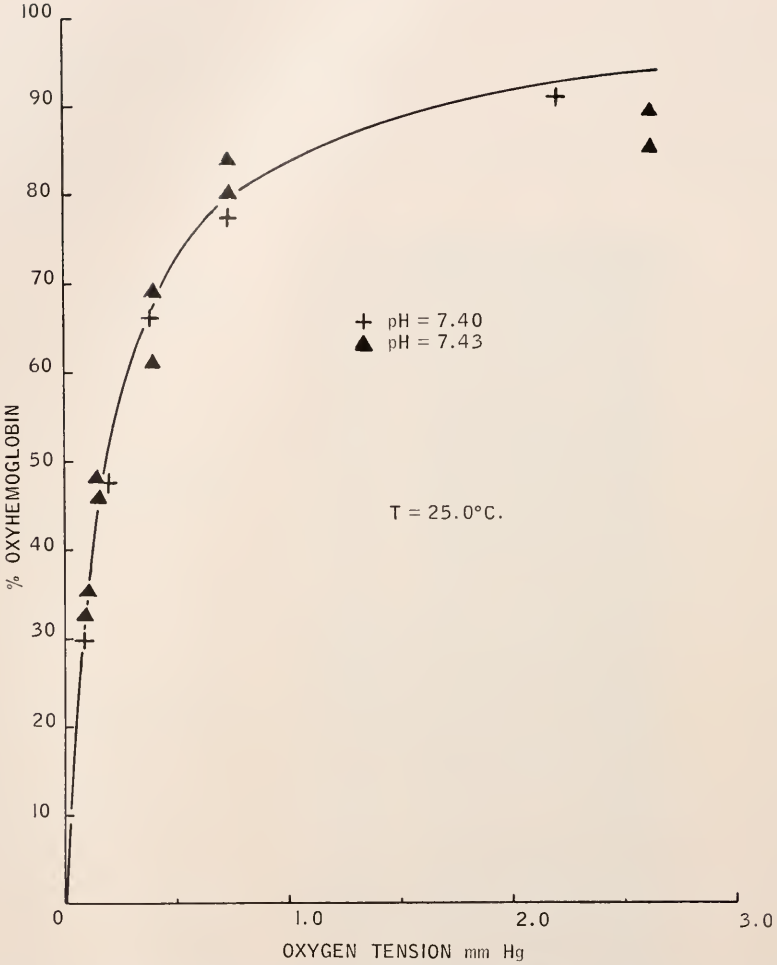

FIGURE 4. Oxygen dissociation curve for *Phacoides pectinatus* hemoglobin. The solid line is drawn for the Hill equation with p_{50} equal to 0.19 mm. Hg and n equal to 1.

ration shifts to 422 mμ (0,2). The *Phacoides pectinatus* preparation absorbs to a greater extent at the β peak than at the α for both oxy- and carboxyhemoglobin.

Oxygen dissociation curve

The results of this section are summarized by Figure 4. The curve shown is drawn from the well-known Hill Equation:

$$y = \frac{(p/p_{50})^n}{1 + (p/p_{50})^n}$$

with p_{50} equal to 0.19 mm. Hg and n equal to 1; y represents the fraction of hemoglobin in the oxygenated form, p the partial pressure of oxygen, p_{50} the partial pressure of oxygen at 50% saturation of the pigment and n a constant which can be considered a measure of heme-heme interaction. A value of n equal to 1 indicates zero heme-heme interaction and a hyperbolic dissociation curve.

Sedimentation constant

Two very fast-moving peaks rapidly formed and swiftly moved across the field of view even before the ultracentrifuge had reached full speed. The hemoglobin peak then formed (see Figure 3) and this in turn split into two peaks, with the slower moving peak appearing to be associated with the hemoglobin, as judged by the optical density changes in the cell. The value of the sedimentation constant $s_{20,w}$ was found to be 2.2×10^{-13} seconds from one determination only.

Histology

Microscopic examination of the unstained cryostat sections showed highly localized patches of yellow-brown pigment scattered throughout the cells of the interior of the gill. The patches were in the form of dense granular masses about 5–7 μ in diameter, with the individual granules ranging from about 0.6 to 1.3 μ in diameter; this is apparent from the photomicrograph in Figure 5.

Examination of the tissue stained for occult iron showed green granular masses

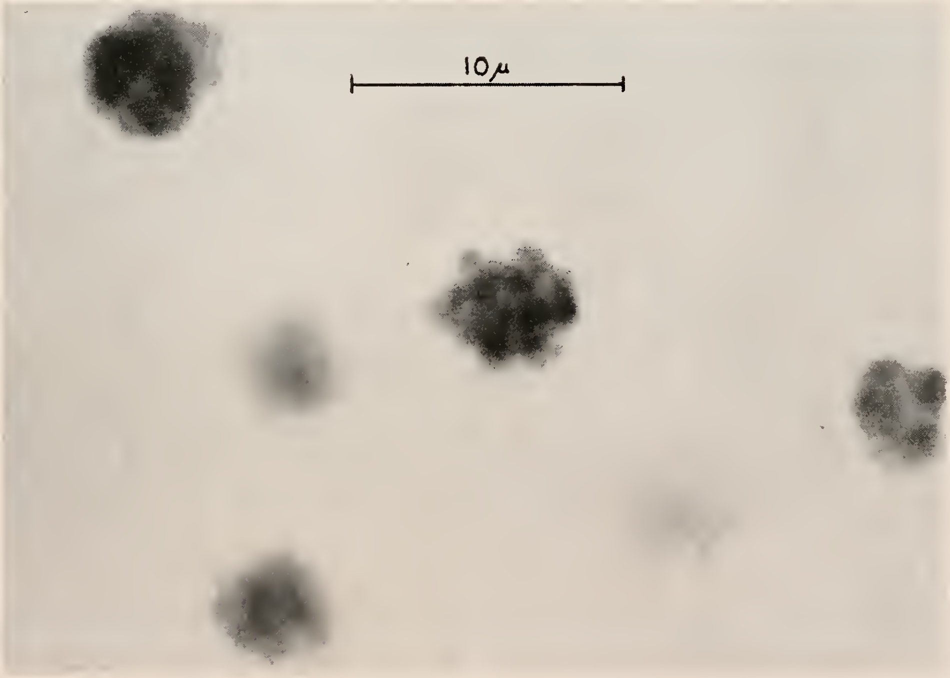

FIGURE 5. Photomicrograph of unstained cryostat section of the ctenidium of *Phacoides pectinatus* taken without a filter in the optical system. The color of the dark masses was yellow-brown.

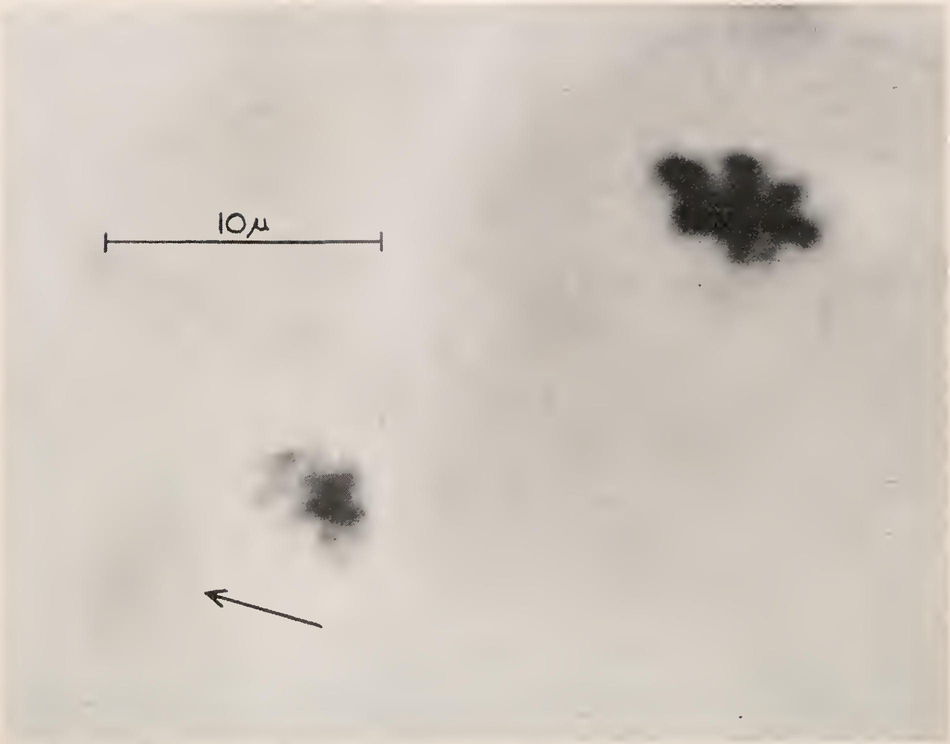

Figure 6. Photomicrograph of section stained for occult iron, taken with a Wratten No. 25 (red) filter in the optical system; the magnification is the same as for Figure 5. The color of the two dense granular masses was green; note the shadowy image of the nucleus at the left-hand side of the picture (arrow).

of approximately the same size and structure as the yellow-brown masses seen in the unstained cryostat section; Figure 6 shows a photomicrograph taken with a Wratten No. 25 (red) filter in the optical system at the same magnification as the photomicrograph of the unstained cryostat section in Figure 5.

The green-staining masses were confined to the interior cells of the gill and were not associated with the ciliated cells of the exterior surface of the ctenidium; this distribution is apparent from examination of Figures 7a, b and c, which are photomicrographs of the same section of the ctenidium taken without any filter, with a Wratten No. 58 (green) and with a Wratten No. 25 (red) filter in the optical system, respectively.

Discussion

Absorption spectra

The behavior of the *Phacoides pectinatus* preparation, with respect to its absorption spectra under various conditions, is very strong evidence in favor of its containing hemoglobin. Unfortunately, the absorption spectra of other lamellibranch hemoglobins appear to be known only for *Arca inflata, Arca subcrenata* and *Tivela stultorum.*

For *Arca inflata* the following wave-lengths for the absorption maxima have been reported: oxyhemoglobin α peak, 578 mμ (Sato, 1931; Kobayashi, 1935), 576 mμ for the purified preparation of Yagi *et al.* (1955a); oxyhemoglobin β peak, 540.8 mμ (Sato, 1931), 541 mμ (Kobayashi, 1935), 540 mμ (Yagi *et al.*, 1955a); deoxyhemoglobin, 559 mμ (Sato, 1931), 556 mμ (Kobayashi, 1935), 555 mμ (Yagi *et al.*, 1955a); carboxyhemoglobin α peak, 573.1 mμ (Sato, 1931), 570 mμ (Yagi *et al.*, 1955a); carboxyhemoglobin β peak, 537.8 mμ (Sato, 1931), 540 mμ (Yagi *et al.*, 1955a).

For *Arca subcrenata*, Kobayashi (1935) obtained values for the wave-lengths of the absorption peaks identical with those for *Arca inflata*. Fox (1953) states that the hemoglobin of *Tivela stultorum* shows sharp absorption bands at 577 and 540 mμ.

With regard to the extent of the absorption at the α and β peaks of oxyhemoglobin, Kobayashi (1935) found that the ratio of the absorption of the α peak to that at the β peak was 0.99±0.02 for *Arca inflata* (?) and 1.00±0.05 for *Arca subcrenata;* Yagi *et al.* (1955a) with a purified preparation of *Anadara inflata* hemoglobin observed values of the millimolar extinction coefficient, ϵ_{mM}, for the α peak of 11.1 and for the β peak of 11.0, thus indicating a slightly greater absorption at the α peak than at the β; this difference was intensified when the carboxyhemoglobin was examined, and here the value of ϵ_{mM} for the α peak was 11.2 and for the β peak 10.8.

The hemoglobin of *Anadara inflata* thus has absorption peaks at wave-lengths only slightly different from those obtained for *Phacoides pectinatus.* However, the two species differ in the values they show for the ratio of absorbency at the α peak to that at the β peak, both for oxy- and carboxyhemoglobin; for *Phacoides pectinatus* this value is slightly less than one, while for *Anadara inflata* the reverse is true.

Oxygen dissociation curve

Manwell (1960a) and Prosser and Brown (1961) have thoroughly reviewed the subject of the function of invertebrate respiratory pigments. In order for a respiratory pigment to play any role in the transport of oxygen from the environment to the tissues, the oxygen tension at which the pigment becomes saturated with oxygen must be of at most the same order of size as the partial pressure of oxygen in the environment; this statement is supported by the innumerable cases in which the oxygen affinities of invertebrate respiratory pigments have been correlated with the ecology of the species studied, such that high oxygen affinities are associated with low environmental oxygen tensions. A state of controversy exists as to whether the pigments serve as reserve stores of oxygen, which come into use only when ambient oxygen levels become low, or whether they serve to facilitate the transfer of oxygen, even when ambient oxygen levels are high enough to maintain the pigment almost entirely in the saturated condition. Those who hold the latter view, point out that the total oxygen capacity of the pigment is too small to constitute an effective reserve. In actuality the pigment probably serves both purposes, facilitating oxygen transport even when environmental oxygen tensions are high and acting as a small reserve store when low, except in the nematodes where the function of their hemoglobin is not understood.

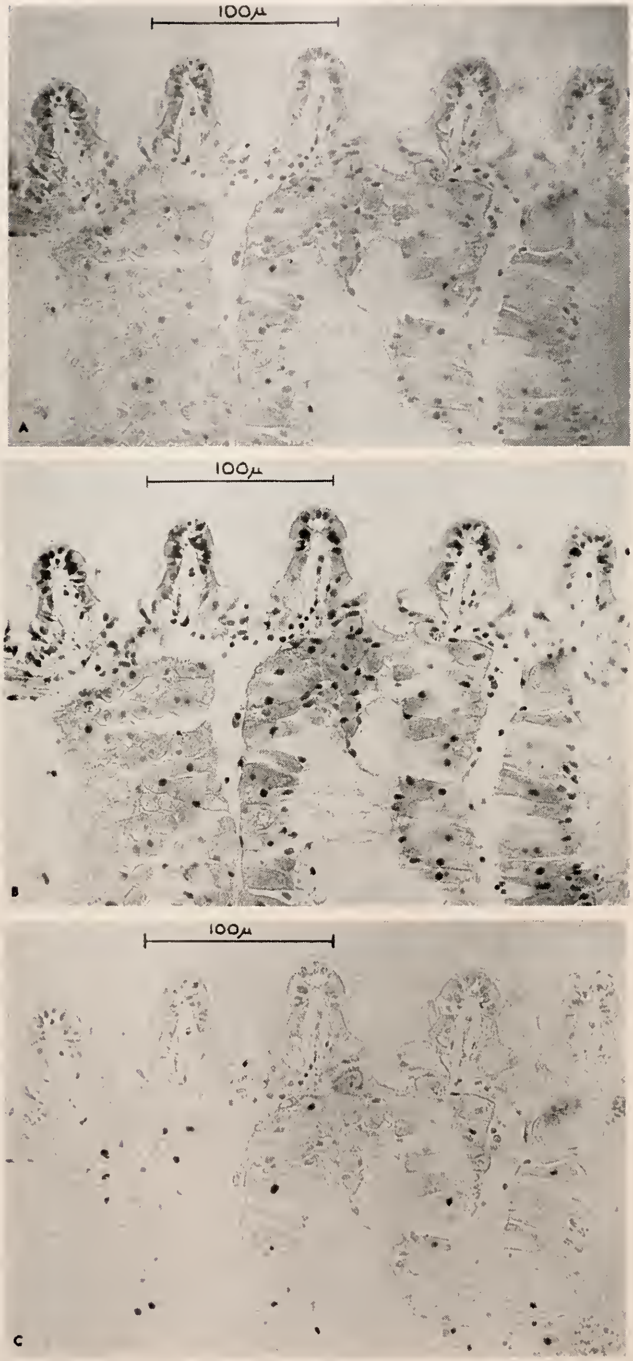

FIGURE 7a. Photomicrograph of section of gill stained for occult iron, taken with no filter in the optical system. Note the dense mats of cilia of the ciliated cells of the surface of the ctenidium.

The oxygen affinity of at least one lamellibranch hemoglobin, that of *Anadara inflata,* has hitherto been determined; working with hemolyzed blood of this species, Kawamoto (1928) found a value for p_{50} equal to 10 mm. Hg at 20± 1° C. and a value for n of 1.155; the determination was made in the absence of CO_2.

Species of *Anadara* are surface-living forms (as far as this author is aware); *Phacoides pectinatus,* on the other hand, lives deep in what are most likely anaerobic muds. The differences in the values of p_{50} reflect the differences in ecology of the two species. The hemoglobin of *Phacoides pectinatus* with its p_{50} of 0.19 mm. Hg is far more comparable to that of the polychaete worm, *Travisia pupa* than to *Anadara inflata.* This worm was studied by Manwell (1960b), and its muscle hemoglobin has a p_{50} of only 0.08 mm. Hg; coelomic hemoglobin, 0.36 mm. Hg; and vascular hemoglobin, a p_{50} varying between 0.53 and 1.10 mm. Hg, depending on pH; this worm lives, like *Phacoides pectinatus,* totally buried in the mud and apparently dies of oxygen poisoning when removed from its substrate and kept in aerated sea water. However, in making interpretations such as these from values of p_{50} or n, obtained from impure or unphysiological preparations of a respiratory pigment, it must be borne in mind that the values for these same parameters obtained for the pure pigment or for the pigment in the intact animal may be different. This was brought out by Manwell (1960c) who showed how values of n changed with the degree of manipulation of the pigment.

Sedimentation constant

The results of the sedimentation determination should be taken with certain reservations, not only because of the impure nature of the preparation, but also because the results have not been extrapolated to zero protein concentration; the possibility also exists that actually it may not have been the hemoglobin peak which was measured.

Prosser and Brown (1961) have published a table giving values of molecular weight and $s_{20,w}$ for a number of respiratory proteins. Vertebrate hemoglobins, excluding those of the cyclostomes, contain four heme groups and associated polypeptide chains, and have molecular weights of about 4 × 17,000; values of $s_{20,w}$ obtained for this type of molecule are about 4.5×10^{-13} seconds. The circulating hemoglobins of the cyclostomes appear to have but one heme group per molecule, molecular weights ranging from 19,100 to 23,100 and values of $s_{20,w}$ ranging from 1.87 to 2.3×10^{-13} seconds.

Sedimentation constants have been measured for the hemoglobins of two other species of lamellibranch. Svedberg and Hedenius (1934) reported values of $s_{20,w}$ ranging between 3.20 and 4.09, with a mean of 3.46×10^{-13} seconds, for the hemoglobin of *Arca pexata* in an impure preparation. Yagi *et al.* (1955a) reported a value of $s_{20,w}$ of 4.6×10^{-13} seconds at pH 7.45 for their purified preparation of

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FIGURE 7b. Same section as Figure 7a, taken with a Wratten No. 58 (green) filter in the optical system. This filter accentuates the red-staining nuclei and pink background in relation to the green-staining iron-containing granules.

FIGURE 7c. Same section as in Figures 7a and 7b but taken with a Wratten No. 25 (red) filter in the optical system. This filter accentuates the green-staining iron-containing granules. Note, by comparing Figures 7a, 7b and 7c, how the green-staining granules are confined to the cells of the interior of the ctenidium and are not associated at all with the cells of the surface of the ctenidium; compare this with the legend under Figure 1.

Anadara inflata hemoglobin; these data were combined with measurements of the diffusion constant to give a molecular weight of 71,000; coupled with data on iron content, which was about 0.31%, this suggests that molecules of *Anadara inflata* hemoglobin are of a size and shape similar to those of the hemoglobins of higher vertebrates.

The value of $s_{20,w}$ of 2.2×10^{-13} seconds obtained for the hemoglobin of *Phacoides pectinatus* suggests that the molecule consists of one heme-polypeptide unit with a molecular weight in the neighborhood of 17,000. The apparent absence of heme-heme interactions in the oxygen dissociation curve is consistent with a molecule that contains but one heme group; in addition, the apparently low molecular weight is consonant with the intracellular location of the pigment (Prosser and Brown, 1961).

Histology

Two-fold lines of evidence are presented for the occurrence of hemoglobin in the form of granular masses within the cells of the interior of the ctenidia of *Phacoides pectinatus*. First, the color of the granules in the cryostat sections strongly suggests hemoglobin, and second, particles of similar size, shape, and structure take the characteristic green color of iron with the ferrocyanide stain.

One of the most striking findings emerging from the histological studies is the fact that the hemoglobin, if such the yellow- or green-staining particles be, does not appear to be associated with the energy-using ciliated cells of the surface of the ctenidium. The localization of the pigment in granules within the cells is similar to the findings of Griesbach (1891) and Sato (1931) for the erythrocytes of certain other lamellibranchs.

The localization of the presumptive hemoglobin within the cell is in accord with its sedimentation characteristics, which indicate that it has low molecular weight, perhaps of the order of 17,000, and would tend to escape by diffusion unless it were confined within a membrane.

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SUMMARY

1. The ctenidia of *Phacoides pectinatus* contain hemoglobin.

2. The hemoglobin appears to be located in the inner tissue of the ctenidia; it does not appear to be associated with the ciliated cells of the surface of the ctenidia.

3. In the cells in which it occurs, the presumptive hemoglobin is highly localized in the form of granular masses, of diameter about 5–7 μ, with the individual granules having diameters ranging from about 0.6 to 1.3 μ.

4. Evidence is presented that the hemoglobin has a sedimentation constant $s_{20,w}$ of 2.2×10^{-13} seconds, but this value needs confirmation.

5. The oxygen dissociation curve of the hemoglobin exhibits no evidence of heme-heme interaction. The pigment has a p_{50} of 0.19 mm. Hg at 25° C. in pH 7.4, 0.2 *M* phosphate buffer in an impure preparation; this value is in accord with the ecology of the species.

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ON PHOTORECEPTOR MECHANISMS OF RETINULA CELLS

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The neurophysiology of photoreception in arthropods has been studied most intensively in compound eyes and dorsal ocelli of insects and the compound eye of the horseshoe crab, *Limulus*. The photoreceptor cells in all of these eyes are quite similar. They are unipolar neurons provided distally with a specialized region of cell membrane, the rhabdomere. The basic similarity in structure suggests a common mechanism of action of these cells. A simple and appealing hypothesis is that the primary photochemical event in all compound eyes and ocelli triggers the same sequence of excitatory processes in the photoreceptor cells, and that corresponding parts of these cells in different eyes play the same roles. However, the complete sequence of excitatory processes has not been worked out for any particular eye. Granted that the above hypothesis is essentially correct, a common mechanism may be discoverable by pooling data from a number of different eyes. A comparative approach will be used here to arrive at a possible common mechanism of photoreceptor cell action in insects and *Limulus*.

Photoreceptor Cells and Retinulas

Photoreceptor cells in insects and *Limulus* occur in groups, or retinulas. Two kinds of retinulas are shown in Figure 1. The one on the left, and all others in this paper, is arbitrarily drawn with four receptor cells. The number of receptor cells per retinula varies from species to species, and even from retinula to retinula within the same eye. For example, retinulas of the dorsal ocellus of the cockroach, *Blaberus craniifer,* contain from two to five receptor cells, three being the most common number; a two-cell retinula from a *Blaberus* ocellus is shown in Figure 2. Retinulas in the compound eye of the housefly, *Musca domestica*, contain 7 receptor cells (Fernandez-Moran, 1958), while those of the compound eye of the damselfly, *Agriocnemis* (Naka, 1961) contain four. Retinulas of compound eyes in Hymenoptera typically contain 8 receptor cells (Hesse, 1908). Each receptor cell has a rhabdomere, and such cells will be called retinula cells henceforth. All of the rhabdomeres occur at cell boundaries inside the retinula.

A rhabdomere consists of tightly packed microvilli formed from a region of limiting membrane of the retinula cell. Some details of this structure are visible in Figure 2, and in numerous other studies (*e.g.*, Goldsmith and Philpott, 1957; Miller, 1957; Fernandez-Moran, 1958). The rhabdomere appears to be diagnostic of arthropod photoreceptor cells. It is the most probable site of the visual pigment for the following reasons: (1) the distal ends of the rhabdomeres lie at the focus of the dioptric apparatus of the ommatidium of apposition-type compound eyes (Exner, 1891; de Vries, 1956; de Vries and Kuiper, 1958); (2) the rhabdomere has a higher refractive index than the material surrounding it, and therefore it "traps" incident light by internal reflection (de Vries, 1956); (3) the cytoplasm closest to the rhabdomere is often packed, as in *Limulus* (*e.g.*, Miller, 1957), with opaque, black pigment granules; (4) the distal ends of the rhabdomeres in the compound eye of the backswimmer, *Notonecta glauca* (Lüdtke, 1953), undergo retinomotor movements, and lie closer to the cornea in the dark-adapted state than in the light-adapted state. In short, the rhabdomeres have a number of properties which seem adaptively appropriate to light-sensitive organelles of the photoreceptor cells, and no other parts of these cells appear to have such properties. Direct proof that the rhabdomeres contain visual pigment has not yet been given.

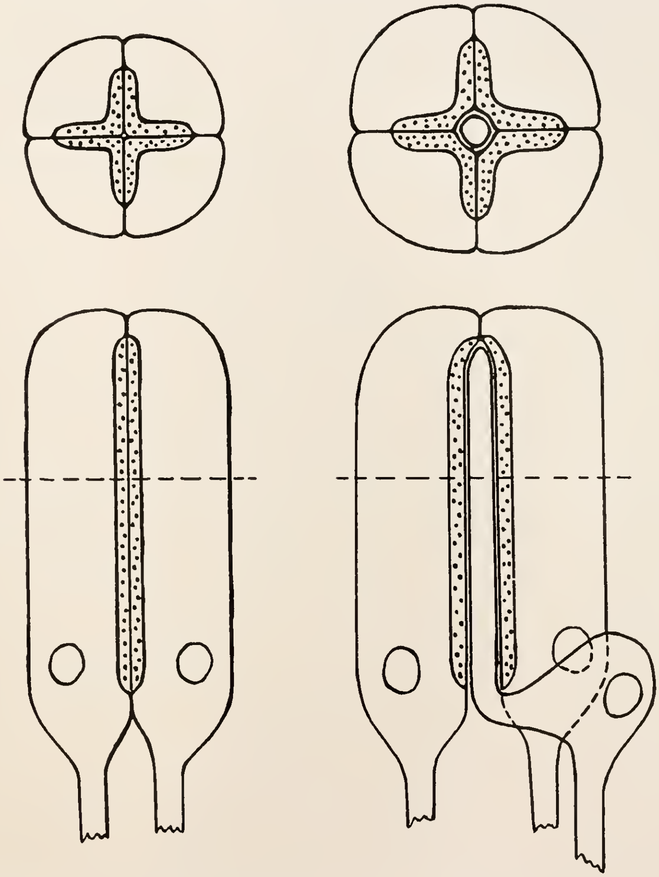


FIGURE 1. Diagrams of two kinds of retinulas. Left: retinula consisting of four retinula cells. Right: an eccentric cell has been added. Rhabdomeres are stippled.

Cells with rhabdomeres are the only kind which have been described in retinulas of insect dorsal ocelli. This is typical for compound eyes of insects as well, except perhaps for the occurrence of rudimentary retinula cells in many Diptera (Dietrich, 1909) and certain other insects (Hesse, 1908). In the compound eye of *Limulus,* however, retinulas contain an "eccentric cell," which has no rhabdomere, in addition to typical retinula cells (Demoll, 1914; Miller, 1957). In the diagram to the right in Figure 1 an "eccentric cell" has been added. The *Limulus* retinula is similar to this diagram except that in *Limulus* there are ten to twenty (Hartline, Wagner and Ratliff, 1956) retinula cells per retinula instead of four.

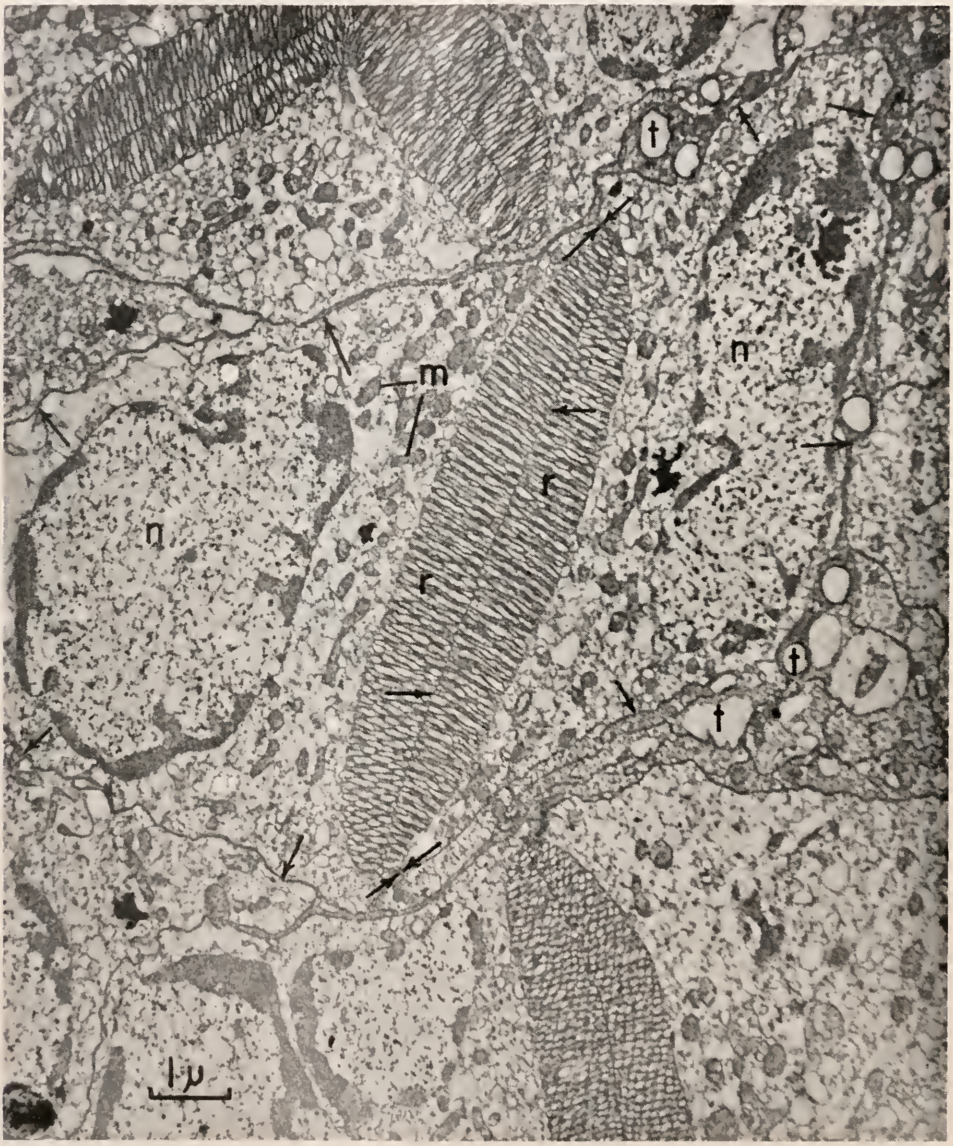
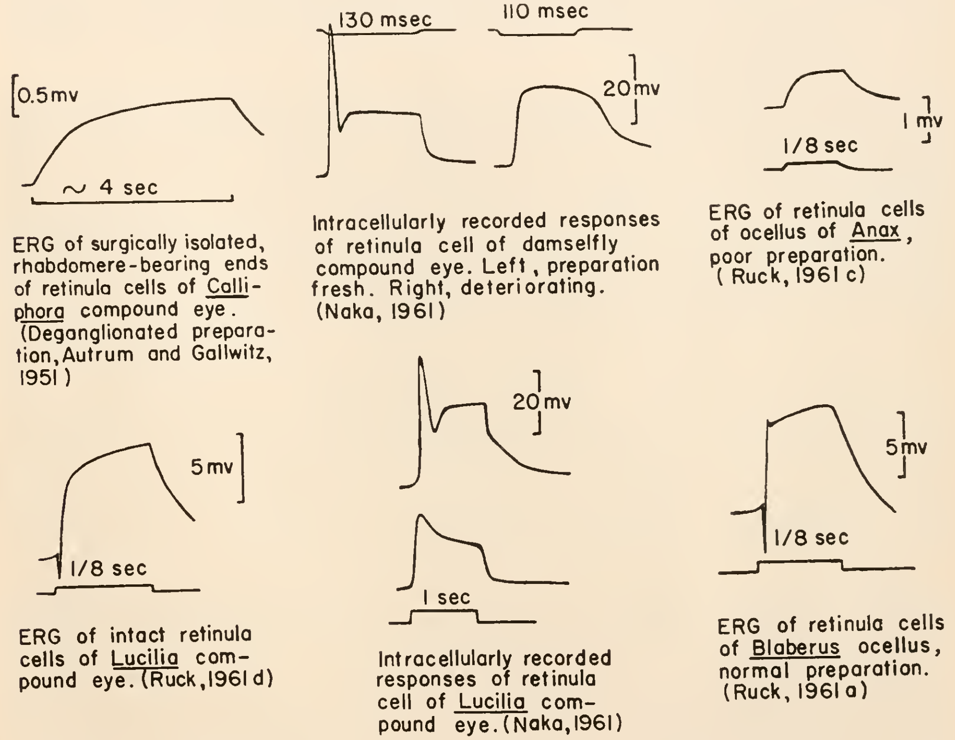


FIGURE 2. Electron micrograph of retinula cells from the ocellus of the cockroach, *Blaberus craniifer*. A two-cell retinula in cross-section appears in the center. Part of a three-cell retinula appears at upper left. Arrows in the two-cell retinula indicate limiting membrane of the retinula cells. Tracheoblasts fill the interstices between neighboring retinulas. Rhabdomere, *r*; nucleus, *n*; mitochondria, *m*; tracheoles, *t*. (From unpublished data of G. A. Edwards and the author.)

Retinula Cell Responses in the Absence of Eccentric Cells

Retinulas without eccentric cells appear to be the simpler kind and their responses to light will be considered first. The ommatidia in compound eyes of grasshoppers (Fernandez-Moran, 1958), dragonflies (Goldsmith and Philpott, 1957), damselflies (Naka, 1961), and dipteran flies (Fernandez-Moran, 1958; Wolken, Capenos and Turano, 1957; Yasuzumi and Deguchi, 1958; Danneel and Zeutzschel, 1957) contain retinulas of this kind. Axons of the retinula cells pass through a fenestrated basement membrane, which forms the inner boundary of the ommatidial layer, and enter the optic ganglion. Several investigators have studied the electroretinograms of compound eyes from which the optic ganglia had been cut or pulled away from the basement membrane (Jahn and Wulff, 1942; Autrum and Gallwitz, 1951; Hartline, Wagner and MacNichol, 1952). This procedure of deganglionation must inevitably remove or damage the axons of the retinula cells, so that the resulting preparation consists essentially of just the ommatidial portions of



ERG of surgically isolated, rhabdomere-bearing ends of retinula cells of *Calliphora* compound eye. (Deganglionated preparation, Autrum and Gallwitz, 1951)

Intracellularly recorded responses of retinula cell of damselfly compound eye. Left, preparation fresh. Right, deteriorating. (Naka, 1961)

ERG of retinula cells of ocellus of *Anax*, poor preparation. (Ruck, 1961 c)

ERG of intact retinula cells of *Lucilia* compound eye. (Ruck, 1961 d)

Intracellularly recorded responses of retinula cell of *Lucilia* compound eye. (Naka, 1961)

ERG of retinula cells of *Blaberus* ocellus, normal preparation. (Ruck, 1961 a)

Figure 3. Responses of insect retinula cells. For ERG's of left and right columns, an upward deflection signifies negativity of an extracellular electrode at the corneal surface of the retinula cell layer. For intracellular recordings of the center column, an upward deflection signifies decreasing negativity of the intracellular electrode, *i.e.*, depolarization of the cell. Of the two intracellularly recorded responses from *Lucilia* (bottom center), the upper one was obtained at 1000 times the stimulus intensity used for the lower.

the retinula cells. All workers agree on the nature of the electroretinogram evoked from deganglionated compound eyes. It is a sustained, monophasic, cornea-negative wave. One of the records of Autrum and Gallwitz (1951), obtained from the deganglionated compound eye of the fly *Calliphora,* is shown in Figure 3 (top left). Similar records have been obtained from the grasshopper, *Trimerotropis* (Jahn and Wulff, 1942), and from the fly, *Musca* (Hartline, Wagner and MacNichol, 1952). The results all indicate that photic excitation results in increased negativity of the extracellular medium at the corneal ends of the retinula cells. A simple interpretation, often made, is that light induces depolarization of the rhabdomere-bearing ends of the retinula cells.

Deganglionated preparations provide no information about responses of retinula cell axons. Dorsal ocelli of cockroaches and dragonflies have been helpful in this regard (Ruck, 1961a, 1961b, 1961c). Retinula cell axons of dorsal ocelli make synaptic contact deep within the ocellus with dendrites of ocellar nerve fibers. Electroretinograms of these organs are quite complex but only two kinds of cells contribute components, retinula cells and ocellar nerve fibers. Components originating in the ocellar nerve fibers can be excluded by recording between the corneal surface of the retinula cell layer and a point deep within that layer. The responses of the retinula cells isolated in this way contain two components (Fig. 3, bottom right). One is a cornea-negative wave essentially like that recorded from deganglionated compound eyes. This event can be isolated in at least two ways, either by bathing the retinula cells in solutions containing high concentrations of potassium ion, or by waiting until a preparation deteriorates physiologically (Fig. 3, top right). The sustained, cornea-negative wave is always the residual component remaining after all other features of the electroretinogram have disappeared. This event, as in the compound eyes, is believed to originate as a depolarization of the rhabdomere-bearing ends of the retinula cells (Ruck, 1961a).

In addition to the sustained, cornea-negative wave, the retinula cell response of the ocellus includes a transient, cornea-positive wave at "on" (Fig. 3, bottom right). Several lines of indirect evidence led to the conclusion (Ruck, 1961a) that the cornea-positive on-component is a depolarizing response of the retinula cell axons. It appears as a negative wave extracellularly in the vicinity of the retinula cell axons. An extracellular electrode at the corneal surface is presumably at the "source" of current flowing to the "sink" in the vicinity of the retinula cell axons. A very similar and presumably homologous component (Fig. 3, bottom left) has been recorded across the intact retinula cell layer of the compound eye of the fly, *Lucilia sericata* (Ruck, 1961d).

It can be deduced from the analysis of the ocellar electroretinogram (Ruck, 1961a) that an intracellular electrode in a retinula cell should record two depolarizing components, one which is sustained throughout the period of illumination, and one which has a transient phase at "on." As yet there are no intracellular recordings from retinula cells of dorsal ocelli, but there are from retinula cells of the compound eye of *Lucilia* (Naka, 1961). In Naka's records, two of which were copied and reproduced in Figure 3 (bottom center), two depolarizing components appear. One is a sustained potential, the other a transient at "on." Naka (1961) recorded similar components from retinula cells in compound eyes of damselflies (Fig. 3, top center), and showed that in retinula cells which are deteriorating physiologically the sustained depolarizing potential appears in isolation. The amplitudes of both components in preparations in good condition are graded with stimulus intensity, and depend upon the state of adaptation (Naka, 1961; Naka and Eguchi, 1962).

Burkhardt and Autrum (1960) have reported intracellular recordings from retinula cells of compound eyes of *Calliphora*. Their records are similar to those of Naka (1961) from *Lucilia*. However, Burkhardt and Autrum feel that the transient event at "on" is not a response of the cell inside of which their electrode is situated. They consider that retinula cells produce only sustained depolarizations, but they have offered no decisive data in support of this interpretation.

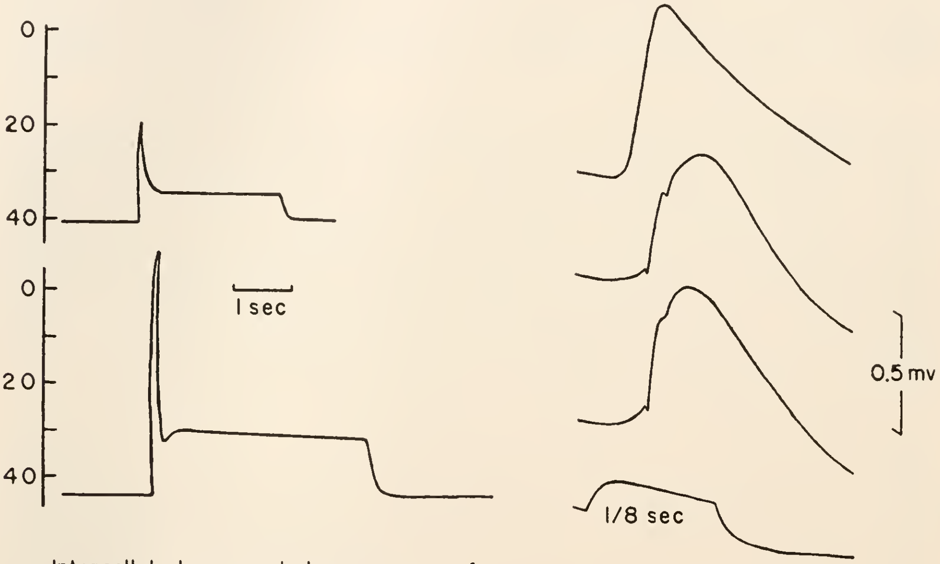
The nature of insect retinula cell responses may be summarized briefly. Results from deganglionated compound eyes establish the rhabdomere-bearing ends of the retinula cells as the source of a sustained, cornea-negative potential in response to light. Intracellular recordings establish that the sustained potential is a depolarization of the retinula cells. Intracellular recordings also indicate a transient, depolarizing on-component. Since this latter event appears as a positive wave extracellularly near the cornea, it cannot originate in the rhabdomere-bearing ends of the retinula cells. If it did it would have the same polarity (negative) extracellularly as the sustained depolarization. The most plausible inference is that the transient on-component is a sign of depolarization of the retinula cell axons.

Retinula Cell Responses in the Presence of Eccentric Cells

In the compound eye of *Limulus*, identification of the responses of retinula cells (those with rhabdomeres) is more difficult than in insects because retinulas of *Limulus* contain eccentric cells. The difficulty is mitigated by two circumstances: (1) the nature of eccentric cell responses is well known because these cells have been impaled with microelectrodes under direct observation (Hartline, Wagner and Ratliff, 1956; MacNichol, 1956); (2) intracellular recordings, unlike those from eccentric cells, but very similar to those from insect retinula cells, have been obtained from retinulas of *Limulus* (Fuortes, 1958; MacNichol, 1956; Benolken, 1961).

A quotation from Benolken (1961) may serve to describe the kinds of intracellular records obtained from *Limulus* ommatidia: "When a micropipette is inserted into a cell of an ommatidium, a resting potential is recorded such that the micropipette becomes polarized about 55 mv negative with reference to an extracellular electrode. As the micropipette is probed through an ommatidium, the electrode may or may not record an electrical response to light. The success or failure of recording a response to light presumably depends upon the location of the micropipette in the photoreceptor unit. If the micropipette has been positioned in a region where an electrical response to light can be recorded, the response takes the form of (a) a graded receptor potential (generator potential) and (b) nerve impulses propagated from the optic nerve. Impulses propagated in the optic nerve appear to be generated near the eccentric cell (MacNichol, 1956). Presumably the electrial activity associated with the propagated impulses is recorded *via* passive conduction through the various structures of the ommatidium when the micropipette is placed in a location which is remote from the eccentric cell.

"The relative amplitudes of the generator potential and the amplitudes of impulse activity which were recorded from the eye were markedly dependent upon electrode placement. In general, whenever the micropipette was positioned so that generator potentials of relatively large amplitude (60 to 90 mv) could be recorded in response to intense illumination, nerve impulse activity of relatively small amplitude (less than 1 mv) was recorded. Conversely, whenever large-amplitude (40 to 50 mv) nerve impulses were recorded, the generator potential amplitude (50 mv or less) was reduced in response to intense illumination."



Intracellularly recorded responses of one kind of cell in ommatidium of Limulus. (Fuortes, 1958)

ERG of compound eye of Limulus.

FIGURE 4. Presumptive retinula cell responses from the compound eye of *Limulus*. Left: the ordinate in Fuortes' records measures the potential in mv of an intracellular electrode relative to an indifferent electrode in the external medium. Negativity is measured downward. Right: (from unpublished records of the author) upward deflection signifies negativity of an extracellular electrode placed just under the cornea. The first of a series of high intensity flashes, ⅛ of a second in duration, elicited the upper response from the dark-adapted eye. Later flashes elicited the next two responses. Each flash produced an illumination of about 10,000 foot-candles (white light) at the cornea. Electrodes were condenser-coupled to the amplifier. The film strip on which the recordings were made moved continuously during the series of flashes.

MacNichol (1956) recognized three types of intracellularly recorded responses from *Limulus* ommatidia: large spikes together with small slow potentials, small spikes with large slow potentials, and small spikes with small slow potentials. MacNichol stated that he had never seen large spikes and large slow potentials together.

Fuortes (1958) stated that in most penetrations of ommatidial cells of *Limulus,* illumination produced large slow potentials and small spikes, spike size ranging between zero and 15 mv. Penetrations of units giving large spikes of 40 mv or more were made more rarely, according to Fuortes.

That large spikes are recorded intracellularly from eccentric cells is known because MacNichol (1956) and Hartline, Wagner and Ratliff (1956) have penetrated exposed eccentric cells under direct observation and obtained large spikes. Waterman and Wiersma (1954) presented strong evidence that axons of eccentric cells are the only ones in the optic nerve of *Limulus* which conduct nerve impulses in response to illumination.

The nature of the retinula cell response in *Limulus* has been more problematical. On the basis of existing information, a reasonable argument can be made that the penetrated units (Benolken, 1961; MacNichol, 1956; Fuortes, 1958) which have given large slow potentials and small spikes, or no spikes at all, in response to illumination are retinula cells. This is intimated by Fuortes (1958) on grounds that this kind of unit is encountered much more commonly than the kind of unit (eccentric cell) which gives large spikes. Two responses of the more commonly recorded type were copied from Fuortes' 1958 paper and appear in Figure 4 (left). The similarity of the responses to those recorded from retinula cells of *Lucilia* and damselflies (Naka, 1961), and *Calliphora* (Burkhardt and Autrum, 1960) is striking. This category of response, in *Limulus* as well as in the insects, includes two depolarizing components, one which is sustained during illumination, and one which is transient at "on."

Benolken's paper is apparently the first in the *Limulus* literature to be concerned entirely with properties of units giving large slow potentials and small spikes. Benolken reports that the transient on-component and the sustained slow potential are graded with stimulus intensity, and that the transient on-component may reverse the membrane potential at very high stimulus intensities. Reversal of membrane potential is apparent also in the records of Figure 4 copied from Fuortes (1958).

Evidence was presented in the previous section of this paper that the sustained component (generator potential) of the insect retinula cell originates in the rhabdomere-bearing end of the cell, the transient on-component in the axon. If this is also the case in *Limulus*, one would expect the sustained depolarization to appear as a negative wave extracellularly near the cornea, with the transient on-component superimposed as a positive wave. That is, one would predict an electroretinogram similar to those recorded from retinula cell layers of insect dorsal ocelli and of the compound eye of *Lucilia* (Fig. 3, bottom right and bottom left). The expectation is borne out as Figure 4 (right) indicates. The electroretinograms shown there were recorded from an intact horseshoe crab which measured about 7 inches across the carapace. One stainless steel electrode was inserted just underneath the cornea, and another was thrust through the carapace into hemolymph about an inch away from the experimental eye. Stimuli of high intensity and ⅛-second duration were presented at rates varying from 1 per second to 1 per 5 seconds for a period of several minutes. The upper record in Figure 4 (right) is the response of the dark-adapted eye. It is a simple, cornea-negative wave, with perhaps a slight inflection at the foot of the ascending limb. The next two responses are those of the partially light-adapted eye and were selected for greatest prominence of the inflections on the ascending limb. Under the stated conditions, the electroretinogram of *Limulus* becomes quite similar to that of the insect retinula cell layer. Duality of the *Limulus* electroretinogram was the subject of a study of Wulff (1950), who suggested that retinula cells and also eccentric cells contribute to the response. An alternative suggestion is that the *Limulus* electroretinogram is generated entirely in the retinula cells, and consists of a generator potential originating in the rhabdomere-bearing ends of the cells, together with a retinula cell axon response. The latter is generally much less conspicuous in *Limulus* than in certain insect eyes, but it can be made fairly prominent with repetitive stimulation at high intensity. The suggestion that a retinula cell axon response occurs in *Limulus* must be reconciled with the evidence that the retinula cell axons in *Limulus* do not conduct nerve impulses (Waterman and Wiersma, 1954).

The Function of the Retinula Cell Axons

The function of the retinula cell axons has been suggested in the case of the dorsal ocelli of dragonflies (Ruck, 1961a, 1961b, 1961c): depolarizing responses of the retinula cell axons cause the release of inhibitory transmitter substance which evokes hyperpolarizing postsynaptic potentials in the dendrites of ocellar nerve fibers; a spontaneous dark discharge of nerve impulses is inhibited as a consequence. Inhibition of ocellar nerve impulses has also been observed in *Locusta* (Hoyle, 1955) and *Calliphora* (Autrum and Metschl, 1961).

Properties of the presumptive retinula cell axon response are well enough documented to attest that it is not a conventional propagating nerve impulse. Its amplitude (*i.e.,* that of the transient on-component) in intracellular recordings from retinula cells of *Lucilia* and damselflies (Naka, 1961; Naka and Eguchi, 1962), and perhaps from *Calliphora* (Burkhardt and Autrum, 1960; their Figure 3), is graded, and depends upon stimulus intensity and the level of adaptation. In *Limulus,* likewise, intracellular recordings from units giving large slow potentials and small spikes show that the transient on-component is graded in amplitude with stimulus intensity, and may even reverse the membrane potential at high stimulus intensities (Benolken, 1961; Fuortes, 1958). These data indicate that this component does not have the properties of propagating nerve impulses.

Tentatively, then, the retinula cell axon response is a local, nonpropagating or decrementally propagating event. If transmission at the first synapses in the optic pathway depends upon such a local event, the distance between the origin of the retinula cell axons and the synapses must be very short. Retinula cell axons in ocelli and compound eyes of insects are indeed very short, less than a mm. in length. The first synapses in the ocellus lie at the base of the ocellar cup, a fraction of a mm. from the cornea (Cajal, 1918; Ruck, 1957). The postsynaptic potential has been described for the dragonfly ocellus (Ruck, 1961a, 1961b). It is a cornea-positive wave associated with inhibition of ocellar nerve impulses, and it is easily interpreted as a hyperpolarizing postsynaptic potential.

In the insect compound eye the first synapses occur in the *lamina ganglionaris,* a complex neuropile situated close to the basement membrane of the ommatidial layer (*e.g.,* Cajal and Sánchez, 1915). Whether nerve impulses occur in second order neurons of the *lamina ganglionaris* has not been definitely established, but postsynaptic potentials have been allocated to the *lamina.* Autrum and Gallwitz (1951) found that a sustained cornea-positive component of the electroretinogram of *Calliphora* could be removed completely by surgical means only when the *lamina ganglionaris* was cut away from the ommatidia. Surgical removal of the *lamina* involves simultaneous removal of the retinula cell axons, as mentioned previously in this paper and elsewhere (Ruck, 1961a, 1961d), and consequently another kind of experiment is needed to discriminate between cornea-positive components which may originate in the retinula cell axons and in the *lamina ganglionaris*. Such an experiment has been performed (Ruck, 1961d) on the compound eye of *Lucilia*. There are indeed two cornea-positive components, one the retinula cell axon response, and the other a sustained potential from the *lamina ganglionaris*. The latter event has the same wave form and polarity as the postsynaptic potential of dragonfly ocellar nerve fibers, and consequently has been identified tentatively as a hyperpolarizing postsynaptic potential in second order neurons of the *lamina ganglionaris* (Ruck, 1961d).

In the *Limulus* compound eye, as in the insects, a synaptic region lies very close to the ommatidia. This is the peripheral plexus formed by the intermingling of collaterals from both retinula cell axons and eccentric cell axons (Ratliff, Miller and Hartline, 1958). Positive identification of the pre- and postsynaptic units has not yet been reported. The plexus mediates inhibitory interactions among neighboring ommatidia. The nature of the interactions has been described very thoroughly (Hartline, Wagner and Ratliff, 1956; Hartline and Ratliff, 1957, 1958). If A and B are neighboring ommatidia, illumination of A alone causes an increased frequency of nerve impulses in the eccentric cell axon from A. Illumination of B alone causes an increased frequency of impulses in the eccentric cell axon from B. The impulse frequency in eccentric cell A is reduced if B is simultaneously illuminated, and *vice versa*. Interactions among neighboring ommatidia are eliminated when the axon collaterals which interconnect them are severed.

In a comparative sense it seems significant that lateral interactions in the *Limulus* compound eye are of an inhibitory nature. It reminds one of inhibition of nerve impulses in ocellar nerve fibers, and the evocation of hyperpolarizing postsynaptic potentials by retinula cells in ocelli, and perhaps also in compound eyes of insects. It suggests that the retinula cells in *Limulus* are the presynaptic units in the peripheral plexus, the eccentric cells the postsynaptic units, and that the retinula cells are performing a general, perhaps evolutionarily primitive, inhibitory presynaptic function in the plexus.

The observations of Tomita (1958) that antidromic electrical stimulation of the optic nerve of *Limulus* produces lateral inhibition do not support the suggestion made here that retinula cells initiate the inhibition, but neither do they refute it. One possible interpretation of Tomita's observations is that antidromic nerve impulses propagating in a given eccentric cell axon enter the peripheral plexus and initiate inhibition at junctions between this axon and neighboring eccentric cell axons, and that orthodromic nerve impulses evoked by illumination in eccentric cell axons act in the same manner. Another possible interpretation is that inhibition of nerve impulses in a given eccentric cell axon is initiated by retinula cells of neighboring ommatidia in two different ways: (1) directly, through the agency of a local potential, and subsequent release of inhibitory transmitter, evoked by the generator potential in the retinula cell axons and their collaterals in the plexus; (2) indirectly, through ephaptic excitation of retinula cell axons and their collaterals by nerve impulses, antidromic or orthodromic, propagating through the peripheral plexus in eccentric cell axons. In their present state, the data on antidromically produced lateral inhibition appear to have no decisive bearing on the suggestion that retinula cells initiate the inhibition.

Major comparative points which have been made are summarized briefly in Table I. The evidence supporting entries in the table may be found in previous parts of the text. Information to replace the two question marks in the table would be most helpful.

Retinula Morphology and the Origin of the Generator Potential

1. *Rhabdomere membrane and distal, non-rhabdomere membrane*

The conclusion that the generator potential originates in the rhabdomere-bearing end of the retinula cell depends upon the evidence (see above) that this event occurs in retinulas which contain retinula cells only, and persists in such retinulas following surgical removal of the retinula cell axons. The rhabdomere-bearing end of the retinula cell is bounded by two morphologically distinguishable regions of limiting membrane (Fig. 2), *rhabdomere membrane,* from which the microvilli of the rhabdomere are formed, and the remainder which may be designated *distal, non-rhabdomere membrane.* Both of these membrane regions must be considered as possible sites of origin of the generator potential. A light-induced change, such as a decrease in membrane resistance, occurring in either region could cause depolarization of the retinula cell. Thus far, neither intracellular nor extracellular recordings have permitted discrimination between electrical properties of the two kinds of membrane. A significant problem probably exists here, and the experimental evidence needed to solve it is likely to be difficult to obtain. There appears to be little doubt that the primary photochemical event occurs in the rhabdomere, or at least in very close association with it. The problem is to determine whether the primary electrical event, the generator potential, occurs in rhabdomere membrane in close proximity with the photochemical event, or in distal, non-rhabdomere membrane some distance away. In the absence of direct evidence bearing upon this problem, it may be worthwhile to suggest a speculative solution based upon indirect evidence. The rest of this section is frankly speculative in nature.

Table I

	Insect ocellus	*Insect compound eye*	*Limulus compound eye*
Retinula cells rhabdomere-bearing ends	depolarizing generator potential	graded, depolarizing generator potential	graded, depolarizing generator potential
axons	depolarizing potential, recognizable as transient on-component	graded, depolarizing potential, recognizable as transient on-component	graded, depolarizing, non-propagating potential, recognizable as transient on-component
Second order neurons	(ocellar nerve fibers)	(*lamina ganglionaris* units)	(eccentric cell axons of neighboring ommatidia)
postsynaptic membrane response	hyperpolarizing postsynaptic potential	hyperpolarizing postsynaptic potential	?
nerve impulses	inhibited by illumination	?	inhibited by illumination

There are data, both morphological and physiological, which suggest the hypothesis that the generator potential originates in distal, non-rhabdomere membrane, and that rhabdomere membrane behaves simply as a fixed resistance through which an appreciable fraction of the current associated with the generator potential may flow. Consider that solitary retinula cells do not occur in insects or *Limulus,* and that rhabdomeres, almost without exception (*e.g.,* see Fig. 6, right), occur at cell boundaries inside the retinula. According to the hypothesis, a solitary retinula cell (Fig. 5, left) undergoing depolarization might be expected to suffer current "leakage" through rhabdomere membrane. It is conceivable that the retinula-type organization (Fig. 5, center) is advantageous because, with rhabdomere facing rhabdomere, current flowing inward through distal, non-rhabdomere membrane is conserved for outward, excitatory flow through membrane of the retinula cell axons.

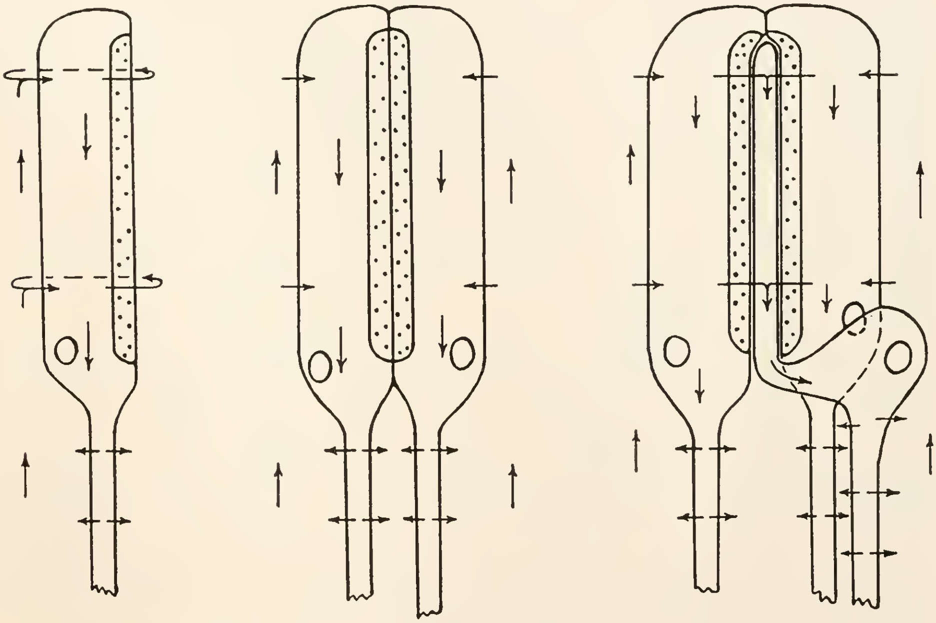


FIGURE 5. Suggested pattern of current flow produced by the generator potential mechanism in three different morphological situations. Arrows indicate direction of movement of positive charge. See text for explanation.

In a retinula (Figs. 1, 2), the rhabdomeres taken collectively are virtually surrounded by distal, non-rhabdomere membrane which forms the circumference of the retinulas as a whole. If distal, non-rhabdomere membrane is the site of origin of the generator potential, while rhabdomere membrane is not directly involved and behaves simply as a fixed resistance, a test probe inserted between adjacent rhabdomeres might be expected to "see" the generator potential as a positive-going wave, much as though the test probe were actually inside a retinula cell. Manipulating a test probe into position between adjacent rhabdomeres would be extremely difficult in most retinulas. Perhaps the technical problem has been simplified somewhat by the existence of a naturally evolved "test probe" in the form of the distal process of the eccentric cell of *Limulus* (Fig. 5, right).

With this thought in mind, some of the data obtained from the *Limulus* ommatidium will be reviewed.

One kind of unit in the *Limulus* ommatidium gives large, depolarizing slow potentials and small spikes, or no spikes at all, in response to illumination (MacNichol, 1956; Fuortes, 1958; Benolken, 1961). It was suggested earlier that this kind of unit is the retinula cell. Another kind of unit gives much larger spikes (MacNichol, 1956; Fuortes, 1958; Benolken, 1961), but smaller, depolarizing slow potentials (MacNichol, 1956; Benolken, 1961); Fuortes (1958) makes no comment concerning the relative size of the slow potential. The latter kind of unit is the eccentric cell, according to the observations of MacNichol (1956) and Hartline, Wagner and Ratliff (1956). The depolarizing slow potential (generator potential) coincides in time with a decrease in resistance measured between an electrode inside an eccentric cell and an electrode in the saline medium bathing the eye (Fuortes, 1959). The magnitude of potential change of the electrode inside the eccentric cell is directly proportional to the magnitude of the resistance change, and both are directly proportional to the frequency of nerve impulses in the eccentric cell axon (Fuortes, 1959; Rushton, 1959). On the basis of these data, Fuortes (1959) and Rushton (1959) suggested that the change in potential of the electrode inside the eccentric cell arises because of a permeability change of eccentric cell membrane, and that this change is most probably produced by a chemical substance released during illumination of the photoreceptor.

The experiments of Fuortes (1959) may be interpreted differently according to one's assumptions regarding the site of the light-induced decrease in membrane resistance. Between the inside of the distal process of the eccentric cell and the external medium (Fig. 5, right) lie eccentric cell membrane, rhabdomere membrane, and distal, non-rhabdomere membrane of the retinula cells. Any one of these could conceivably be the site of the resistance change measured by Fuortes (1959). If eccentric cell membrane is the site, it is reasonable to assume that the retinula cells release a substance which increases the permeability of eccentric cell membrane, and that the generator potential originates in the eccentric cell. However, this interpretation forces one to seek another explanation of the origin of the generator potential in retinulas which lack eccentric cells.

A quite different interpretation emerges if two assumptions are made: (1) that the resistance change in *Limulus* is restricted to distal, non-rhabdomere membrane; (2) that there is, in effect, no intercellular space between rhabdomere and distal process of the eccentric cell. The distal process of the eccentric cell may then be regarded as a passive structure, so situated that it acts as a pathway for a fraction of the current which flows inward through distal, non-rhabdomere membrane during the interval that that membrane is occupied by the generator potential. In completing the circuit, current flows outward through soma or axon of the eccentric cell and excites the discharge of nerve impulses there. This idea is consistent with the observation (MacNichol, 1956; Benolken, 1961) that the generator potential appears smaller in intracellular recordings from (eccentric) cells giving large spikes than from cells giving small spikes, or no spikes at all; if the generator potential originates in distal, non-rhabdomere membrane of the retinula cell, it is reasonable to expect that a fraction of it be "dropped" across the resistances represented by rhabdomere membrane and eccentric cell membrane. If this interpretation is correct, no light-induced resistance change should occur between an electrode in the eccentric cell and an electrode in a retinula cell of the same retinula. An experiment to test this prediction has not yet been reported.

If the rhabdomere is the site of the primary photochemical event, and distal, non-rhabdomere membrane is the site of the change in ionic permeability which is responsible for establishing the generator potential, it is reasonable to suggest that the rhabdomere releases a substance which traverses the interior of the retinula cell to reach distal, non-rhabdomere membrane and alter its permeability.

2. *Variations in retinula morphology and their possible significance*

If it is plausible that the eccentric cell of *Limulus* is excited by currents generated in the retinula cells, it is equally plausible that in retinulas without eccentric cells, currents generated in one cell flow through all of the cells. In other words, functional interactions among retinula cells of the same retinula must be considered a definite possibility. In many insects, for example, in dipteran flies (Dietrich, 1909), the rhabdomeres are separated by a space in the center of the retinula. A retinula with such a central space is represented diagrammatically in Figure 6 (left). Certainly the separation of the rhabdomeres increases the optical isolation of the individual retinula cells, and may consequently improve the visual acuity of the eye (de Vries, 1956), but the space between the rhabdomeres may also act as a shunt path for currents flowing into it through the rhabdomeres, and thus may decrease electrical interactions among the retinula cells.

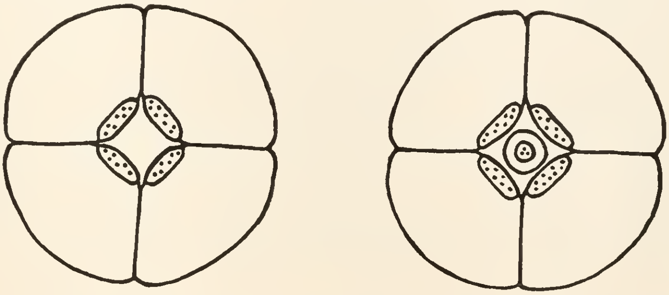


FIGURE 6. Two variations in retinula morphology found in insects. Left: like Figure 1 (left) except for space in center of retinula. Right: a retinula cell with an "internal" rhabdomere is added. See text for explanation.

In at least one insect, *Notonecta glauca* (Lüdtke, 1953), one retinula cell differs from its neighbors in that it has an "internal" rhabdomere and occupies a central position in the retinula (Fig. 6, right). It would be most interesting to know whether the "internal" rhabdomere is formed by invagination of outer membrane of the retinula cell. The internal position of the rhabdomere may have the significance that it prevents "leakage" of excitatory current (the kind of leakage suggested for the hypothetical, solitary retinula cell in Figure 5, left), and reduces electrical interactions between this retinula cell and its more typical neighbors. The existence of an "internal" rhabdomere incidentally suggests that the photochemical event, if it occurs in the rhabdomere, leads to excitation of distal, non-rhabdomere membrane; in this case the rhabdomere appears to be surrounded by distal, non-rhabdomere membrane. More information is needed concerning the structure of the central retinula cell of *Notonecta*.

Finally, the argument that neighboring retinula cells may interact electrically induces caution in interpreting intracellular recordings from retinula cells. It is quite conceivable that a microelectrode situated inside one retinula cell, or even in the central space of some retinulas, actually records activity of all the cells of the retinula.

RECAPITULATION

A common mechanism of action has been suggested for retinula cells of insects and *Limulus*. The rhabdomere is assumed to be the site of the primary photochemical event in the photoreceptor process, but the generator potential is thought to originate some distance away in distal, non-rhabdomere membrane of the retinula cell. The generator potential is considered to evoke a graded, depolarizing, non-propagating response in the retinula cell axons. The retinula cell axon response is thought to cause the release of a chemical transmitter substance which mediates synaptic transmission at the junctions between the retinula cells and second order neurons of the optic pathway. The general, perhaps evolutionarily primitive, synaptic relationship between retinula cells and the second order neurons is thought to be an inhibitory one, exemplified by the dorsal ocellus of the dragonfly (Ruck, 1961a, 1961b), in which spontaneous nerve impulse activity of second order neurons is inhibited by illumination of the retinula cells. This relationship is thought to be preserved in the peripheral plexus of the *Limulus* compound eye where the eccentric cell is inhibited by (retinula) cells of neighboring ommatidia. The distal process of the eccentric cell, a structure which to present knowledge has no counterpart in insect retinulas, is thought to lead off a fraction of the current produced by the generator potential mechanism of the retinula cells. This current, flowing outward through membrane of the soma and/or the proximal portion of the eccentric cell axon, is thought to excite the discharge of eccentric cell nerve impulses; that is, excitation of the eccentric cell is considered to depend, in effect, upon electrical synaptic transmission. Waterman and Wiersma (1954) pointed out that retinula cell axons in *Limulus* do not conduct nerve impulses, and that therefore the eccentric cell, which is not itself a photoreceptor, is essential to the transmission of information to the central nervous system. To their discussion may be added the suggestion that the evolutionary forerunner of the *Limulus* eccentric cell might have been a spontaneously active postsynaptic neuron, which was inhibited by illumination of the retinula cells. A new growth from the soma of this postulated ancestral neuron could have invaded the retinula to become the distal process of the eccentric cell. With this addition, the nerve impulse frequency of the eccentric cell axon could be increased by illumination, whereas without the distal process the impulse frequency could only be decreased. Evolutionary adjustments of threshold of the eccentric cell to its own autoexcitatory mechanism, to electrical currents generated by the retinula cells, and to inhibitory transmitter substance released by retinula cell axons, might have combined to produce the physiological properties of the present eccentric cell of *Limulus*.

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THE STRUCTURE AND METABOLISM OF A CRUSTACEAN INTEGUMENTARY TISSUE DURING A MOLT CYCLE[1]

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"And the body form is moulded by the epidermis. It is the epidermis which shapes the organism in all its details; the other tissues, which support and nourish and connect one part with another, follow the lead which the epidermis gives. Even the great integrating systems, the endocrine organs and the central nervous system, are historically a part of the ectoderm, and where they influence the body form they do so chiefly by the activation of the epidermis."

In these few sentences Wigglesworth (1945, p. 23) outlined one of the great challenges of arthropod physiology. It is known that during the period preceding ecdysis the arthropod epidermis undergoes profound changes in structure which probably reflect the synthesis of a new exocuticle (or exoskeleton) to encompass the reshaped and enlarged animal (Kuhn and Piepho, 1938; Travis, 1955, 1958; Wigglesworth, 1933). The preparation for molting is also accompanied by a 50 to 1900% increase in oxygen consumption by the whole animal (Bliss, 1953; Edwards, 1950, 1953; Nyst, 1941; Poulson, 1935; Schneiderman, 1952; Schneiderman and Williams, 1953; Scudamore, 1947), which means that the metabolism of some or all of the tissues is vastly increased.

This paper describes the structure and metabolism of the integumentary tissue of the land crab, *Gecarcinus lateralis,* during the molt cycle. Integumentary tissue is comprised of two sheets of epidermal cells separated by a layer of connective tissue.

As a source of integumentary tissue, the branchiostegites, sheets of tissue which form the covering of the branchial chambers, were selected for several reasons. A small piece of tissue, 10 to 20 mm.^{2}, could be excised from each branchiostegite without affecting the length of the molt cycle. Routinely, two samples were taken from the same animal at different times in the molt cycle. At its maximum, the integumentary tissue of the branchiostegites is only 450 μ thick. Without being sliced it should, therefore, permit adequate diffusion of oxygen to interior cells (Field, 1948). The use of a tissue which did not have to be sliced reduced to a minimum changes in oxygen consumption due to loss of coenzymes by diffusion from injured cells or by the action of nucleotidases (Mann and Quastel, 1941).

Drach (1939) subdivided the crustacean molt cycle into stages A through D, depending on the state of exoskeleton. The exoskeleton was pliable in stages A and B, 4 to 8 days immediately following ecdysis, whereas stage C, the three-month intermolt period, was marked by a rigid exoskeleton. The 15- to 30-day premolt period, during which the two outer layers of the new exoskeleton were formed, was designated as stage D.

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The cytological changes of the epidermis and other cells of the integumentary tissue of *Gecarcinus* were correlated with the exoskeletal changes described by Drach. Metabolic studies of the integumentary tissues were then undertaken. The rates of oxygen consumption of pieces of integumentary tissues taken from a series of animals in each stage of the molt cycle were measured. The exact stage of each animal was determined from inspection of sections of tissue removed from the animal on the day of the metabolic studies.

Limbs autotomized from crustaceans are regenerated during the premolt period (Bliss, 1956). In the present study, when the size of regenerating limb buds was correlated with the structure of the epidermis, it was found that regeneration of limbs is complete before any morphological changes are detected in the epidermis.

A report on some of these results has appeared previously (Skinner, 1958).

MATERIALS AND METHODS

1. *Selection and maintenance of animals*

Specimens of the land crab, *Gecarcinus lateralis*, collected in Bermuda or Bimini, were maintained in the laboratory as described by Bliss (1953). Animals ranging in carapace width from 3.5 to 5 cm. were used. At each feeding period (*i.e.*, every ten days), regenerating limb buds were measured. During the premolt period, they were measured more frequently.

2. *Removal of tissue*

Animals were anesthetized by chilling at 4° C. for 15 to 20 minutes. A piece of tissue approximately 3 mm. by 4 mm. was removed from one branchiostegite and the opening in the branchial chamber was covered by a piece of plastic sealed in place by melted paraffin. Operated animals were returned to their individual containers and observed until external signs of an approaching ecdysis were seen (*i.e.*, growth of regenerating limb buds, swelling of pericardial sacs (Bliss, 1953, 1956); depressibility of the exoskeleton (Drach, 1939)). A second piece of tissue was then removed from the other branchiostegite, the crab being similarly treated and observed until ecdysis. The time from tissue removal until ecdysis was thus known. These data, coupled with the histological condition of the tissue, permitted the determination of the duration of each stage of the premolt period.

3. *Histological and histochemical methods*

Pieces of tissue were fixed in Bouin's solution, dehydrated in ethanol, imbedded in paraffin and sectioned at 7 to 10 μ. Sections were stained with either Mallory's triple stain or phosphotungstic acid.

RNA was visualized by staining with dilute solutions (0.01%) of methylene blue over a pH range of 3 to 6.2. In that pH range, most basic staining is attributed to nucleic acids, since the carboxyl group of proteins has a pK of 2 and is not dissociated (Swift, 1955). Control sides were subjected to RNase[3] hydrolysis before methylene blue staining. For contrast, the sections were counterstained with dilute eosin.

The periodic acid-Schiff method was used to demonstrate the presence of glycogen. Control sections in this series were pretreated with salivary amylase. The tissues of 75 animals were studied.

4. *Preparation of tissues*

Pieces of tissue (50 to 100 mg. wet weight) were cut from the branchiostegites. During the intermolt period, the epidermis is tightly attached to the innermost region of the exoskeleton, the membranous layer (Fig. 1), which can be separated as a thin sheet from the outer region of the exoskeleton. To avoid disrupting the epidermis, intermolt tissues were removed with the membranous layer attached. During that part of the premolt period (stage D_1 and later) when the membranous layer is being resorbed, the remainder of the old exoskeleton can be lifted away from the integumentary tissues. Pieces of isolated tissue, with membranous layer (intermolt), without membranous layer (early premolt), or with newly synthesized exoskeleton (late premolt and early postmolt) were weighed on a Roller Smith torsion balance and immersed in 0.5 ml. iced *Carcinus* perfusion fluid. They were then blotted on filter paper and placed in the Warburg vessels.

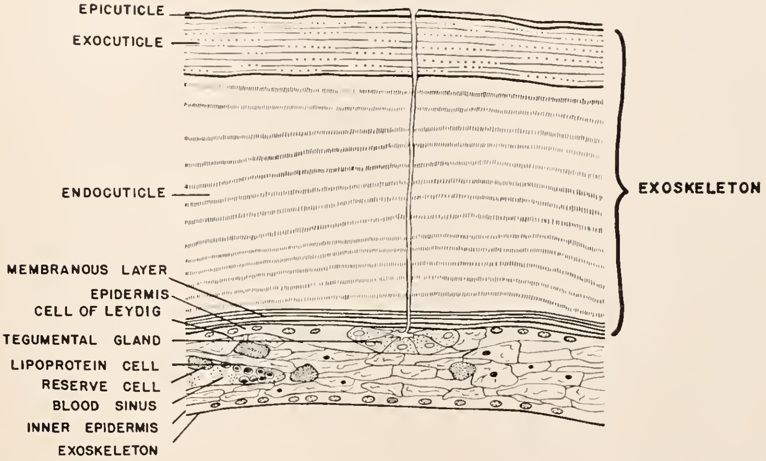

FIGURE 1. Diagrammatic cross-section of the integumentary tissue of an intermolt *Gecarcinus,* drawn to scale. The epidermal layer described is the one adjacent to the thick outer exoskeleton. 100 × magnification.

5. *Oxygen consumption measurements*

The oxygen consumption of pieces of tissue was determined manometrically. The main chamber of five-ml. Warburg vessels received buffered (0.02 *M* Tris, pH 7.7) *Carcinus* perfusion fluid (Pantin, 1946) containing 15 γ streptomycin and 4 γ penicillin per ml. The center well contained 0.1 ml. of a 10% solution of potassium hydroxide. Dinitrophenol and Krebs substrates (Krebs, 1950), when added, were placed in the sidearm. To each 0.75 ml. was added 0.25 ml. of a solution containing the following substrates in milliequivalents/liter: 4.9 pyruvate, 4.9 glutamate, 5.4 fumarate, 9.2 glucose. In experiments testing the effect of cyanide, potassium hydroxide was replaced by 0.1 ml. of a calcium hydroxide-potassium cyanide suspension, of the concentration required to saturate the gas phase at the desired molarity (Robbie, 1948). Calcium hydroxide was used as the alkali in control vessels.

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[3] The following abbreviations are used: RNase, ribonuclease; Tris, trishydroxymethylamino methane; PAS, periodic acid-Schiff; DNP, dinitrophenol.

TABLE I

Schedule of premolt and early postmolt events in Gecarcinus

Stage	Initiation (days before ecdysis)	Completion (days before ecdysis)	Event
D_0	25+	?	Gastrolith formation
D_0	25	5 to 10	Regeneration of autotomized limbs
D_1	12	10	Resorption of old exoskeleton, beginning with the membranous layer; increase in height of epidermal cells to 10 μ.
D_1	10	8	Further enlargement of epidermal cells to 30 μ, separation from old exoskeleton by resorption of membranous layer.
D_2 (early)	7	5	Formation of two-layered epicuticle
D_2 (late)	4	2	Formation of exocuticle
D_3	1	0.5	Slight decrease in size of epidermal cells
D_4	0.5	0	Blood pink
Ecdysis			
A	0	1	Epidermal cells shrink slightly
B	1	5	Formation of endocuticle, about 7 μ each day
C_1 and C_2	5	?	Formation of endocuticle continued, at the same rate

The total volume, including the tissue, was 1 ml. The flasks were incubated at 25° C. and shaken at the rate of 130 oscillations per minute.

At the end of the experiment, tissues were rinsed in distilled water, blotted on filter paper and dried in a 100° oven for 24 hours. They were then weighed on a Sartorius balance. The rate of oxygen consumption (Q_{O_2}) was expressed as μl. O_2/mg. dry weight hour. At least two aliquots of tissue were taken from each animal.

Results

1. *The molt cycle*

The duration of the molt cycle of a mature *Gecarcinus lateralis* (carapace width 3 cm. or greater) is four to six months (Table I). The intermolt period, C_1 to C_4, comprises all of the cycle except for a 30-day premolt period (D_0 through D_4) and a short postmolt period (A through B) when synthesis of the exoskeleton continues. In the premolt period, animals regenerate autotomized limbs, resorb more than three-fourths of the old exoskeleton and synthesize an exoskeleton to replace the one lost at ecdysis.

During the premolt period, the weight of animals increased by 13 to 30% of the intermolt value, due to the absorption of water. After ecydysis, animals weighed one-half as much as during the preceding intermolt period. Within 10 days after ecdysis, they had regained the weight lost at ecdysis and an additional increment due to growth, which occurs only during the early postmolt period when the exoskeleton is still pliable. After each ecdysis, there was a 1 to 7% increase in carapace width and a 6 to 22% increase in weight.

2. *Cytology of the integumentary tissue*

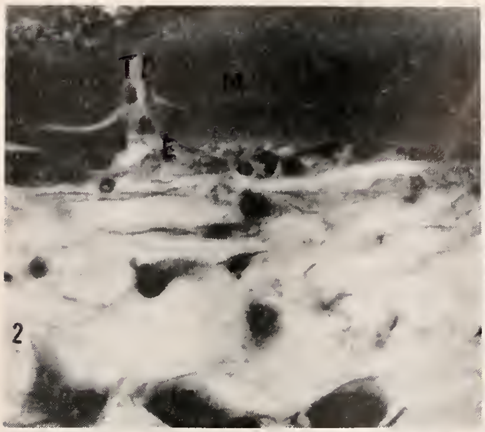
The branchiostegites are bounded on their inner and outer surfaces by single sheets of epidermis (Fig. 1). The epidermal layer bounding the inner surface of the branchiostegites synthesizes a 7-μ-thick layer of cuticle with staining characteristics similar to those of the two-layered epicuticle. The outer epidermal layer, on the other hand, synthesizes the thick exoskeleton, composed of a 7-μ epicuticle. a 30-μ exocuticle and a 200- to 400-μ endocuticle. Both epicuticle and exocuticle are synthesized during the premolt period, while the endocuticle is formed during the postmolt period. In this study, attention has been directed to the structural changes of the outer epidermal layer.

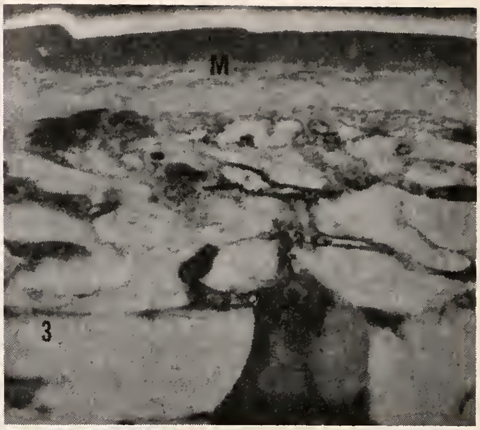
Between the two epidermal layers there is a layer of connective tissue, the bulk of which is composed of cells of Leydig (Cuénot, 1893). Among the cells of Leydig are scattered reserve cells (Hardy, 1892) and small blood sinuses which contain lipoprotein cells (Sewell, 1955). At the inner edge of each epidermal layer there are tegumental glands whose secretory cycle is not correlated with the molt cycle, since both replete and empty glands are present at all stages of the molt cycle.

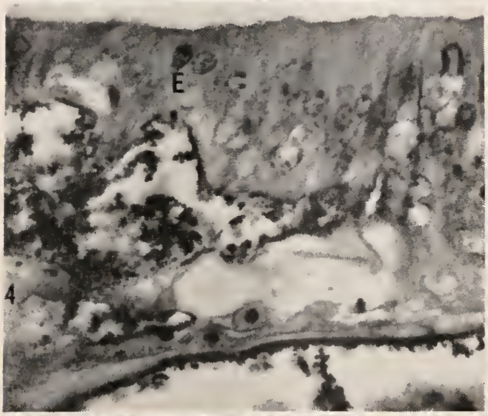
3. *Cytological changes of the integumentary tissue*

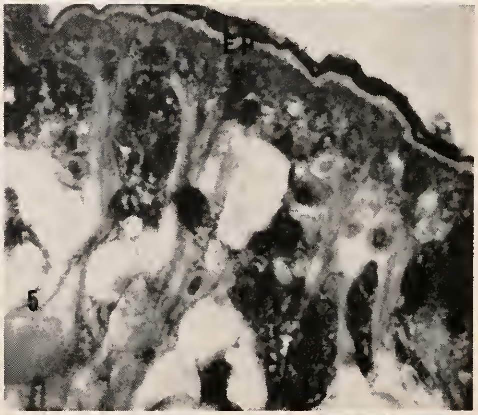
a. Epidermis

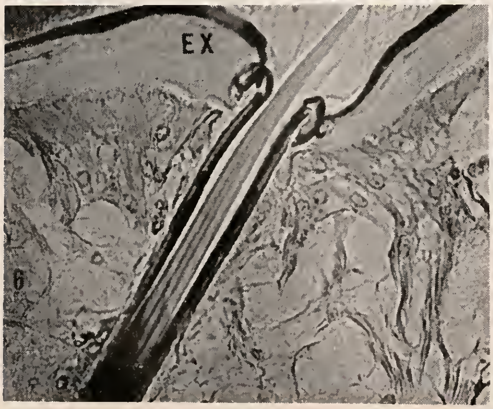
Integumentary tissue removed from an animal 16 days before ecdysis (Fig. 2) is identical to that from an intermolt animal (Fig. 1). Resorption of the membranous layer, the innermost region of the exoskeleton, begins approximately 11 days before ecdysis, and the nuclei of the epidermal cells have enlarged (Fig. 3). As the membranous layer is digested, its staining characteristics change. Intact membranous layer is PAS-negative while partially digested membranous layer is PAS-positive, indicating that a material with adjacent hydroxyl groups is made











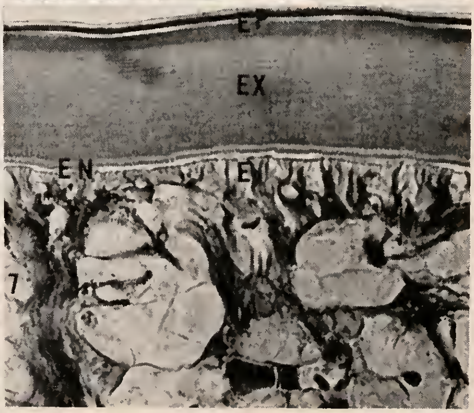


FIGURE 2, Stage D_0. Integumentary tissue from an animal 16 days before ecdysis. Only the nuclei of the epidermal cells (E) are visible beneath the membranous layer (M) of the exoskeleton. The duct of a tegumental gland (TD) can be seen entering the exoskeleton. Note that the tissue is identical to tissue from an intermolt animal (Fig. 1).

FIGURE 3, Stage D_1. Integumentary tissue from an animal 11 days before ecdysis. The membranous layer (M) is being resorbed.

available for oxidation by HIO_4 and for consequent reaction with the leucho-fuchsin dye. The nature of the reactive material is unknown. However, it is known that the crustacean exoskeleton is composed of approximately equal amounts of chitin, which is PAS-negative, and protein (Lafon, 1948). Part of the protein may be a mucoprotein with a carbohydrate component possessing adjacent hydroxyl groups.

Eight days before ecdysis there is complete separation of the exoskeleton from the epidermal cells which have enlarged further (Fig. 4). Synthesis of both layers of the epicuticle has been completed by the fifth day preceding ecdysis (Fig. 5), and the 30-μ-thick exocuticle is formed during the following two days (Fig. 6). The endocuticle, whose formation begins on the second day following ecdysis, is thickened at the rate of 7 μ per day (Fig. 7) for at least a week (the period of time during which samples of tissue were taken).

b. Other integumentary cells

Near the end of the intermolt period, the number of lipoprotein cells increases. The cytoplasm of these cells becomes dotted with acidophilic granules during the early premolt stages. As ecdysis approaches, the cells increase their granular contents and move to the epidermis. By the time the epicuticle has been completed, near the end of the premolt period, the lipoprotein cells have disappeared. Their disappearance, coupled with the similar staining characteristics of the outer epicuticle and the lipoprotein cell granules, leads to the speculation that the granules are incorporated into the epicuticle. The changes of the lipoprotein cells of *Gecarcinus* parallel those of the homologous cells of the green crab (Sewell, 1955) to this point. However, in the green crab the small granules coalesce to form one large droplet immediately preceding ecdysis. In *Gecarcinus*, the granules do not coalesce; rather, large cells with homogeneous cytoplasm, similar to the reserve cells described by Hardy (1892), are seen at all stages of the molt cycle.

c. Glycogen metabolism of the integumentary tissue

As can be seen in Table II, the glycogen content of the outer epidermal layer changes markedly during the premolt period. As the old exoskeleton is broken down and new exoskeleton synthesized, there is an increase in glycogen content of the epidermis. The glycogen content of the cells of Leydig also increases before and decreases after ecdysis, suggesting that these cells serve as intermediates in glycogen metabolism, probably receiving glucose from the blood and releasing it to the epidermis.

FIGURE 4, Stage D_1. Integumentary tissue from an animal 8 days before ecdysis. The epidermal cells (E) are greatly enlarged and are completely separated from the old exoskeleton.

FIGURE 5, Stage $D_{2\ (early)}$. Integumentary tissue from an animal 5 days before ecdysis. The epidermal cells (E) have completed synthesis of both layers of the epicuticle (EP).

FIGURE 6, Stage $D_{2\ (late)}$. Two-layered epicuticle completed; exocuticle (EX) partially formed. Hair follicle visible.

FIGURE 7, Stage B. Integumentary tissue from an animal two days after ecdysis. Epicuticle, exocuticle as above. First layers of endocuticle (EN) seen. Epidermal cells have decreased in size and their nuclei are no longer visible.

TABLE II

Glycogen content of cells of the integumentary tissue

Stage	Epidermal cells			Cells of Leydig	Lipoprotein cells	
	Size		Glycogen content	Glycogen content	Number	Contents
	Height, μ	Width, μ				
Intermolt C_4	4	10–17	+	+	++++	– – –
Premolt D_0	4	10–17	+	+	+++++	
D_1	10	10–17	+	++	+++	+
D_2 (early)	30	10–17	++	+++	++	++
D_2 (late)	100	10–17	+++	+++	+	+++
D_3	80	10–17	++	+++	+	++
D_4	80	10–17	+	++	+	++
Postmolt A	20	10	+	+	–	
B	10	10	+	+	–	

4. *Formation of gastroliths*

Gecarcinus stores calcium resorbed from the old exoskeleton as concretions (gastroliths) which form in the lining of the stomach. In *Gecarcinus*, gastrolith formation begins about 30 days before ecdysis. Within three days after ecdysis, the gastroliths have disappeared completely.

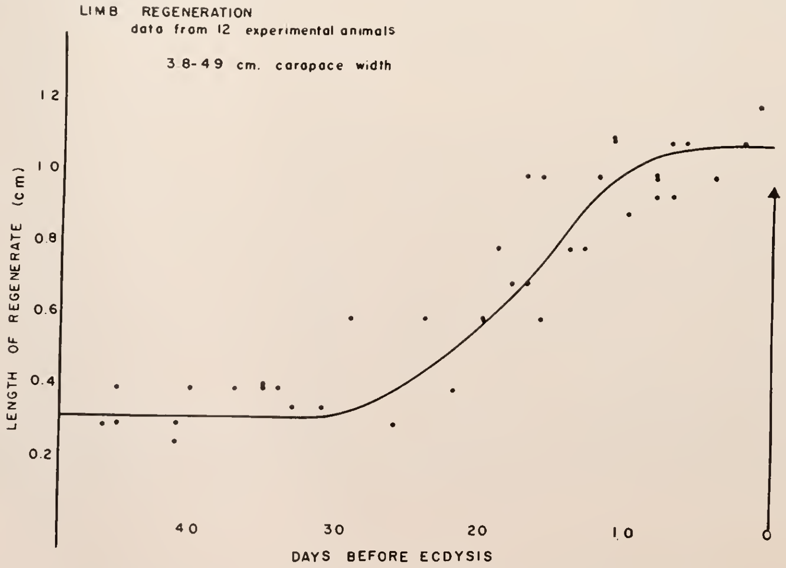


FIGURE 8. Compilation of growth curves of regenerating limbs of 12 *Gecarcinus*. Note plateau until 25 days before ecdysis, when limb buds begin to grow again. Limb bud reaches maximum size approximately 10 days before ecdysis.

5. *Regeneration of limbs*

Within the first two to three weeks after a limb is autotomized, a small limb bud, 2–4 mm. long, grows out from the scar tissue which forms over the stump of the autotomized limb. The limb bud remains in this form until the succeeding premolt period when it resumes growth (Bliss, 1956).

In Figure 8, the length of regenerating limbs of animals used in this study is plotted against time. It can be seen that about 30 days before molt, limb buds begin to elongate, that they grow at a rapid rate for approximately 20 days, completing their growth about 10 days before ecdysis.

6. *Oxygen consumption of the integumentary tissue*

a. Rate of oxygen consumption at each stage of the molt cycle

The integumentary tissues of intermolt, early premolt (stages D_1, $D_{2\ \text{early}}$) and early postmolt (stage B) consume oxygen at approximately equal rates (Table III; Fig. 9). The Q_{O_2} of integumentary tissue synthesizing the 30-μ-thick exocuticle ($D_{2\ \text{late}}$) is significantly higher than that of intermolt tissue (Table III; Fig. 9). The Q_{O_2} of tissue removed from two animals immediately after ecdysis (stage A) is also significantly higher than that of intermolt tissue.

TABLE III

The mean Q_{O_2} of the integumentary tissue at each stage of the molt cycle

Stage	Number of animals	Mean Q_{O_2}	Standard deviation
C_4	8	0.53	0.14
D_0	5	0.30	0.14
D_1	7	0.49	0.19
D_2 (early)	3	0.46	—
D_2 (late)	11	0.85	0.28
A	2	0.72	—
B	6	0.38	0.08

The mean Q_{O_2} of tissues removed from animals in stages D_1, $D_{2\ \text{early}}$, and B has been tested statistically against the mean Q_{O_2} of tissues removed from C_4 animals. They have been found not to differ significantly. However, the mean Q_{O_2} of tissues removed from D_0 animals is significantly lower than that of the tissue from intermolt animals. No explanation can be given for this decrease in respiratory rate at the initiation of the premolt period. The mean Q_{O_2} of tissues removed from animals in stage $D_{2\ \text{late}}$, when the exocuticle is being formed, is significantly higher than the mean Q_{O_2} of tissues from animals at all other stages.

b. Effect of cyanide and dinitrophenol

Both 10^{-4} and 10^{-5} *M* cyanide inhibited oxygen consumption of integumentary tissues from intermolt and premolt animals by 60 to 95%. As seen in Figure 10, 10^{-4} and 10^{-5} *M* DNP increased the oxygen consumption of the integumentary tissues.

c. Effect of endogenous substrates in blood serum and of Krebs substrates

Tissues bathed in *Carcinus* perfusion fluid (Pantin, 1946), which was 25% (v/v) in *Gecarcinus* blood serum, respired at a greater rate than tissues bathed in the salt solution alone. The increase was in the order of 50 to 200%.

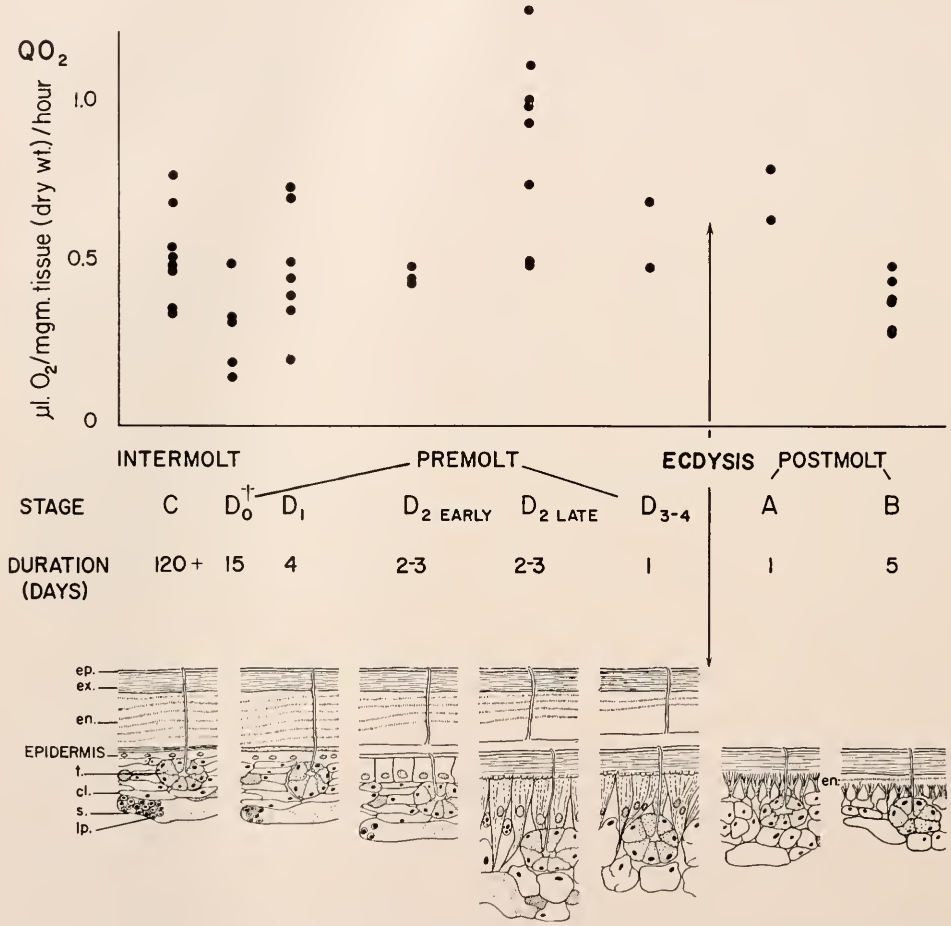


FIGURE 9. The Qo_2 and cytology of the integumentary tissue of *Gecarcinus* at each stage of the molt cycle. In stage D_0, the cytology of the integumentary tissue is the same as in the intermolt period. ep = epicuticle; ex = exocuticle; en = endocuticle; t = tegumental gland; cl = cell of Leydig; s = blood sinus; lp = lipoprotein cell.

Attempts at replacing the unknown stimulating components of blood serum with Krebs' substrates (Krebs, 1950) produced only minor increases in oxygen consumption (14 to 30%).

DISCUSSION

As can be seen in Table I, morphological evidence indicates that the first 15 days of the 30-day premolt period in *Gecarcinus lateralis* are devoted to limb regeneration and gastrolith formation. However, it is obvious that during this first portion of the premolt period the integumentary tissues, which retain their intermolt morphology, are active in resorbing calcium from the exoskeleton and allowing its passage to the blood for storage as gastroliths in the stomach lining. Additional evidence of the catabolic activity of the integumentary tissues, preceding any change in their structure, is seen as the membranous layer of the exoskeleton is resorbed.

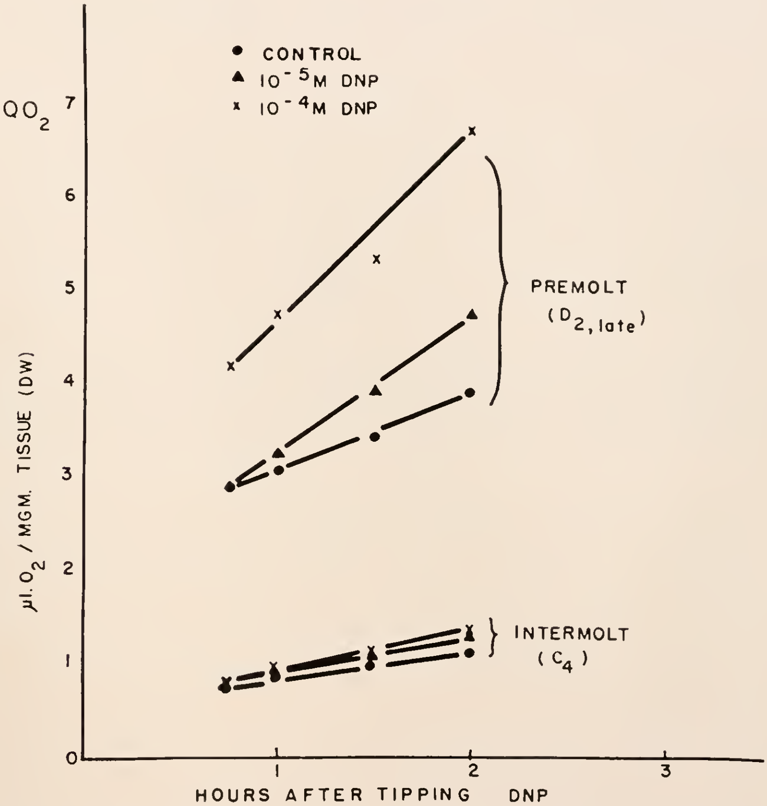


FIGURE 10. The effect of DNP on intermolt and premolt integumentary tissues. After a three-hour incubation period, DNP was tipped from the sidearm into the main vessel.

The time course of events as found in *Gecarcinus lateralis* has been fitted into the stages of Drach (1939) in Table I. Stage D_1, marked by the resorption of the membranous layer of the exoskeleton, occurs 11 days before ecdysis. Synthesis of epicuticle on the seventh day before ecdysis signals the beginning of Stage D_2. On the fourth day before ecdysis, exocuticle formation begins. This stage has been called $D_{2, late}$ to distinguish it from $D_{2, early}$ because it is during $D_{2, late}$ that the oxygen consumption of the integumentary tissues increases. D_3 and D_4, 1.5 days immediately preceding molt, are marked by no further synthesis of exoskeleton. There is, however, some reduction in the size of the epidermal cells. The blood of D_3 and D_4 animals has lost the characteristic blue color of crustacean blood and assumed a pink tinge, due to astaxanthin resorbed from the old exoskeleton (Skinner and Krinsky, unpublished observations).

The increased rate of oxygen consumption of integumentary tissues in $D_{2,\text{late}}$ has been attributed to the rapid synthesis of exoskeleton. The 30-μ-thick exocuticle, composed of approximately equal parts of chitin and protein, is synthesized in this two-day period.

The author would like to express her deep appreciation to her sponsor, Dr. John H. Welsh, for his helpful discussions during the course of this work.

SUMMARY

1. The morphological changes undergone during the molt cycle by the integumentary tissue of the land crab, *Gecarcinus lateralis,* have been described.

2. The time course of limb regeneration and gastrolith formation has been correlated with the morphological changes of the integumentary tissue. The period of premolt activity during which limb regeneration and gastrolith formation occurs precedes the changes in the integumentary tissues and has, therefore, been called D_0.

3. The oxygen consumption of the integumentary tissues has been measured at each stage of the molt cycle. It has been found to increase at the time of synthesis of the exocuticle. The effects of cyanide, dinitrophenol and added substrates on the oxygen consumption of the integumentary tissue have been studied.

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UPTAKE OF ORGANIC MATERIAL BY AQUATIC INVERTEBRATES. I. UPTAKE OF GLUCOSE BY THE SOLITARY CORAL, FUNGIA SCUTARIA[1]

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The suggestion that naturally occurring dissolved organic material may contribute to the nutrition of aquatic animals is usually associated with the name of Pütter (1909). This hypothesis was critically reviewed by Krogh in 1931. He concluded that no firm evidence could be adduced to support it.

In recent years little work has been undertaken which bears directly on this possibility. Collier *et al.* (1953) reported the retention of an unidentified carbohydrate by oysters. Fox and his co-workers in a series of papers (1952, 1953) suggested that dissolved organic material may be adsorbed on inorganic particles of colloidal dimensions and become available to filter-feeding animals in this way. These workers have suggested that such colloidal micelles may comprise a considerable fraction of the "dissolved" organic material normally occurring in sea water. Goldacre (1949) and Cheesman (1956) have argued that protein monolayers at the air-water interface may be significant as food sources for tadpoles and snails.

It is apparent that this work (with the possible exception of that of Collier's group) is not really concerned with dissolved organic material in the sense of small organic molecules in true solution as a potential source of nutrition for aquatic animals. Rather, physical processes of adsorption are invoked to produce colloidal micelles or a denatured monolayer, which then is available to the animal. There is no reason to doubt that such devices do indeed operate. However, the significance, if any, of materials in true crystalloid solution remains to be assessed.

Recent work in our laboratory (Stephens and Schinske, 1961) demonstrated the uptake of several amino acids from dilute solution by a variety of marine invertebrates. However, the concentration of acids employed for most of this work was very high compared to concentrations of organic materials in natural waters. Furthermore, the observations were totally dependent on measuring the rate of disappearance of materials and attempting by suitable control procedures to implicate the animal concerned as the agent. The present work was undertaken, using C^{14}-labelled compounds, in order (a) to permit use of lower concentrations of added organic material, and (b) to provide unambiguous evidence that such material was entering the experimental animal.

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[2] Hawaii Marine Laboratory Contribution No. 170.

Any dissolved organic compound which may be available to marine organisms must be present at very modest concentrations, since the total dissolved organic material of sea water is reported as a few milligrams per liter. The ability to deal effectively with at least some species of organic molecules in extremely dilute solution is a critical consequence of the idea that dissolved material may contribute to the nutrition of aquatic animals. However, demonstration that a particular compound is available to an animal from dilute solution is merely consistent with this hypothesis.

The observations to be reported were undertaken to determine whether the solitary coral, *Fungia,* could remove glucose from dilute solution at a significant rate. They were extended to provide information concerning the mechanisms of uptake. Preliminary reports of this work have appeared (Stephens, 1960a, 1960b).

Materials and Methods

The solitary coral, *Fungia,* was selected as an experimental animal for several reasons: (a) it is locally abundant on coral reef flats fringing Oahu, is easily collected, and is a hardy organism in the laboratory; (b) individuals are sufficiently large to permit easy experimental manipulation; and (c) individuals produce large quantities of mucus which can be readily collected. Animals were collected in the vicinity of the Hawaii Marine Laboratory. Individuals were maintained in running sea water until they were employed in experiments.

The basic procedure involved the addition of measured amounts of uniformly labelled glucose-C^{14} (or other labelled material) to a measured volume of sea water. The movement of this material was followed by monitoring the radioactivity of the ambient sea water and of suitable extracts. Radioactivity was measured using a thin-window Geiger tube. Quadruplicate samples of 0.5 ml. were dried on aluminum planchets and the time for 2000 counts measured. Extracts of the animals were prepared by boiling with dilute NaOH. In the case of all such extracts and solutions, suitable concentration curves were established to correct for differences in self-absorption by the dried samples. All values are corrected for background.

Observations were made on animals maintained in 100 to 200 ml. of sea water. Observations were not continued beyond 24 hours under such conditions. Animals survived such confinement for at least ten days and there was no mortality in the course of the observations.

Oxygen consumption was measured using the Winkler method. During such measurements, the animals were kept in darkness because of the presence of symbiotic algae.

Results

Figure 1 illustrates the disappearance of radioactivity from the solution in a typical set of observations. Initial glucose concentration in this case was 10 mg./liter. Wet weight of the animal was 33.0 grams, temperature 25° C., and the volume of solution was 200 ml. Streptomycin (50 μg./ml.) added to the ambient sea water did not modify this rate of disappearance. Controls, consisting of a blank container with a similar volume of solution, and the skeleton of *Fungia* in a glucose solution, showed no change in radioactivity over a period of 24 hours.

Recovery of radioactivity from a digest of the animals was complete. Digests

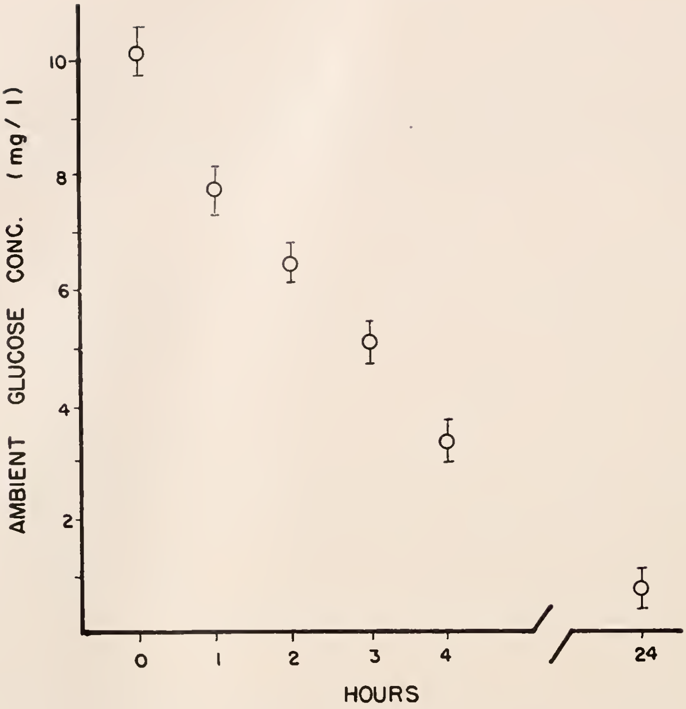

FIGURE 1. Decrease in glucose as measured by radioactivity of the ambient sea water. Volume is 200 ml., weight of animal is 33 grams, temperature 25° C.

were made by boiling in dilute NaOH. After correction for the lower absorption of radiation by the digest, good agreement was obtained between the loss of radioactivity from sea water and its appearance in the animal (Table I). A 5-ml. sample of the ambient sea water solution was acidified. The resulting CO_2 was trapped in a drop of KOH. Counts of this trapped material were not significantly different from

TABLE I

Distribution of radioactive material after four hours. Initial glucose concentration 1.25 mg./liter, temperature 25° C.

Animal	A	B
Weight		
Wet	34.2 gm.	17.0 gm.
Ash	26.7	13.3
Total activity in sea water		
Initial	18,700 c.p.m.	18,700 c.p.m.
Final	4,600	9,100
Activity of digest (corrected for absorption)	14,900 c.p.m.	10,200 c.p.m.
Recovery	104.4%	103.1%

the background rate. Hence, no significant quantity of $C^{14}O_2$ had been produced during the four hours of observation.

Figure 2 is a graph of the log of wet weight in grams against the log of the rate of glucose uptake. Uptake is expressed as milligrams/hour/individual. The open circles represent the uptake of animals in 400–800 ft. candles' illumination at an initial sugar concentration of 5.0 mg./liter. The regression line calculated for these points has a slope of 0.54 and a standard error of 0.09.

The crosses represent the uptake of sugar at an initial concentration of 5.0 mg./liter in darkness. Hence, light intensity is not a factor in rate of uptake.

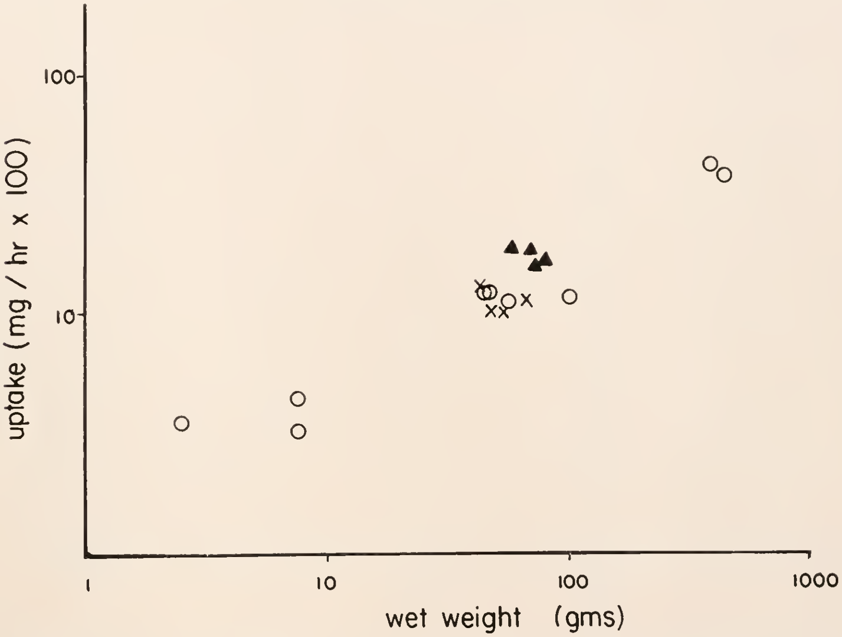

FIGURE 2. Log uptake expressed as mg./hr./individual times 100, plotted against the log of the wet weight. Open circles represent animals in light, crosses are animals in darkness, triangles are animals whose mouths have been plugged with paraffin.

Table II provides data relevant to the relation between initial concentration and the rate of uptake. Animals used weighed 30 to 45 grams. Initial concentrations are listed, together with the amounts of glucose removed in one hour, based on disappearance of radioactive material from the solution. Concentrations above 10 mg./liter were obtained by adding unlabelled glucose. The lowest initial radioactivity was employed in the measurements at 1.0 mg./liter, which was 5.3 ± 0.68 counts/minute. The values for 0.37 mg./liter were obtained with glucose of higher specific activity, so that initial activity exceeded background (21.5 ± 1.1 c.p.m.). Although expressed as uptake/hour, the uptake at high concentrations of sugar was measured over a period of four or twenty-four hours. This was necessary at the highest concentrations because of low percentage rates of upake, and convenient at other concentrations. From such primary data, two methods of calculation are apparently appropriate. If the rate of uptake is linearly related to ambient concentration, the concentration would decrease exponentially, a fixed percentage being removed per hour. This assumption is consistent with the data at ambient concentrations below 20 mg./liter. At high concentrations uptake is apparently independent of concentration. Hence, hourly uptake would most appropriately be estimated by simply dividing total uptake by time. This was done for ambient concentrations greater than 60 mg./liter.

TABLE II

Relation between ambient glucose concentration (S) and rate of uptake (V). Weight is 30 to 45 grams.

S (mg./l.)	V (mg./hr.)	S (mg./l.)	V (mg./hr.)
0.37	0.03	30.0	0.83
	0.02		0.91
1.0	0.03	40.0	1.28
	0.06		1.46
	0.05		0.92
	0.04		0.84
1.25	0.07		0.92
	0.04	50.0	0.79
4.0	0.23		1.17
	0.25		
	0.30	60.0	1.39
	0.20		0.96
5.0	0.19	100.0*	1.90
	0.22		0.90
	0.21		1.93
	0.21		2.23
10.0	0.60	110.0*	0.99
	0.32		1.19
	0.46		0.59
	0.44		
20.0	0.65	200.0*	0.83
	0.50		0.76
	0.60	500.0*	1.46
	0.68		1.56

* Estimated, assuming uptake was linear.

A regression line, calculated by the least squares method for the concentration range 0.37 to 20 mg./liter, has a slope of 0.042 with a standard error of 0.008 (Fig. 3). A regression line for the concentration range 40 to 500 mg./liter has a slope of 0.0012 and a standard error of 0.0009. This is not significantly different from zero, indicating that uptake is independent of concentration in this range. One may conclude that the rate of uptake for animals in this size range is 4.2 ± 0.8% of the ambient glucose per hour at low concentrations and reaches a maximum of 1.20 ± .43 mg./hour (the average of uptake over the range 40 to 500 mg./liter.[3]

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[3] The general form of the relationship of velocity of uptake to concentration of ambient glucose suggests that an adsorptive step is rate-limiting. An alternate form of data presentation is a plot of the reciprocal of the rate of uptake against the reciprocal of ambient glucose concen-

It is of interest to consider the energy requirements of these animals, as indicated by their oxygen consumption. Table III lists the oxygen consumed per hour for 18 animals. The equivalent amount of glucose is also tabulated. The slope and standard error of a regression line relating the log of oxygen consumption and log wet weight is 0.45 ± 0.16. Although the standard error is large, the slope is significantly different from zero. The slope may be used to calculate mean oxygen consumptions at weights of 30 to 45 grams. These are, respectively, 0.383 and 0.472 ml. O_2/hour. These are in turn equivalent to 0.514 and 0.633 mg. of glucose oxidized per hour. At the mean rate of uptake calculated for this size range above, ambient glucose concentrations of 12.2 to 15.0 mg./liter would suffice to provide these amounts of sugar. Furthermore, since the rate of uptake is related to size in the same fashion as is oxygen consumption, ambient concentrations of glucose in this general range should suffice to account for oxygen consumption regardless of size.

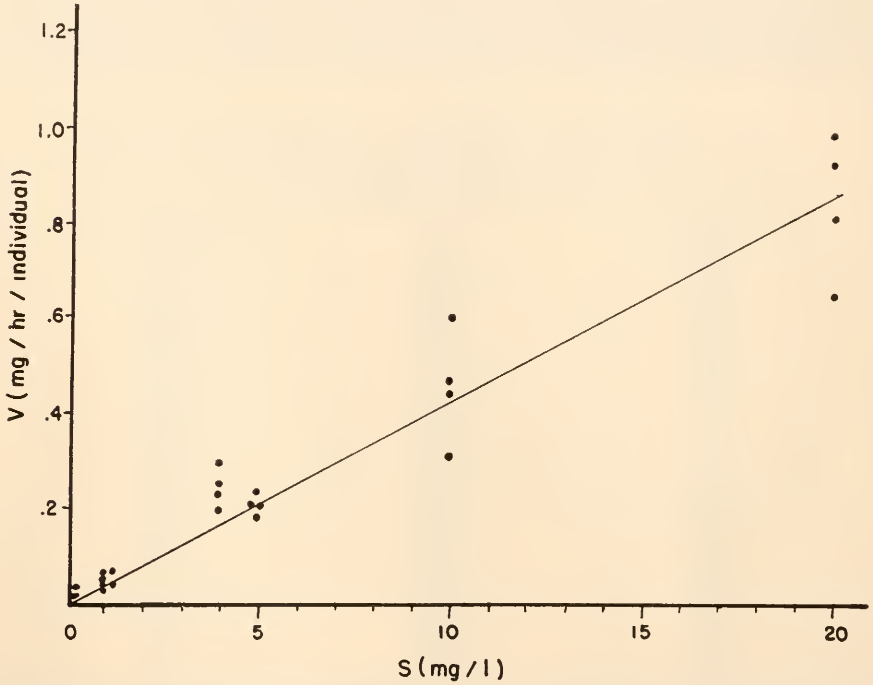

FIGURE 3. Rate of uptake as a function of glucose concentration. The line is the least squares regression line. Weight is 30–45 grams.

The general form of the curve relating concentration and the rate of glucose

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tration. This should approximate a straight line. The regression line of such a plot has a slope of 16.94 ± 0.85 and an intercept of 1.21 ± 0.31. This is formally equivalent to an enzyme-catalyzed reaction and V_{max} and K_m (the concentration at which the velocity is half maximal) can be evaluated. V_{max} is 0.83 mg./hr. and K_m is 20.5 mg./liter. This is in reasonable agreement with the rates calculated more directly from the data. Since departures from linearity for double reciprocal plots are quite common (Neilands and Stumpf, 1958), the presentation in the text is preferable.

uptake corresponds to a Langmuir isotherm. This form suggests the possibility that an adsorptive step in the uptake process might be rate-limiting. Evidence was sought to evaluate two variants of this possibility.

The first hypothetical mechanism considered was the adsorption or binding of glucose to mucus, with subsequent recovery of mucus by the animal. This was suggested by Collier *et al.* (1953) in connection with the retention of carbohydrate by oysters, and has been proposed (Rao and Goldberg, 1954) as a mechanism for uptake of calcium in other invertebrates, including sea anemones. A similar mechanism was also suggested by Korringa (1952) to account for selective retention of phytoplankton by oysters. Finally, Stephens and Schinske (1961) allude to this as a possible but unsupported explanation for the uptake of amino acids they observed.

TABLE III

Oxygen consumption of Fungia as ml. O_2/hr./individual and as equivalent amounts of glucose

Wet weight	ml. O_2/hr.	mg. sugar/hr.
52	0.575	0.770
20	0.384	0.515
53	0.645	0.865
53	0.660	0.884
85	0.795	1.065
59	0.577	0.774
51	0.550	0.736
33	0.365	0.489
62	0.550	0.736
58	0.412	0.552
47	0.362	0.485
64	0.400	0.536
35	0.362	0.485
64	0.563	0.754
56	0.400	0.536
52	0.613	0.820
53	0.587	0.785
55	0.425	0.570

Fresh mucus was obtained by simply collecting the copious flow from one or more *Fungia* in a beaker. The possibility of adsorption was tested directly by filtering mucus after incubation with labelled material. A Millipore filter, type VM (pore size 50 mμ), was employed. Fresh mucus samples could be coagulated by heat, alcohol, and acetic acid, and gave a strongly positive test for carbohydrate with anthrone. The filtrate was negative to these tests. The radioactivity of the filter disc then served as a measure of the material bound to the mucus, which was retained.

Ten ml. of mucus were incubated for 30 minutes with glucose in sea water. Radioactivity was 665 ± 15 c.p.m. After filtering, the material retained was washed with 20 ml. of sea water. After drying, the filter disc showed 8.7 ± 0.6 c.p.m. Since the initial glucose concentration was 8.3 mg./liter, the radioactivity of the filter disc implies the retention of 0.054 micrograms glucose as well as the mucus in the 10-ml. sample. Total mucus solids were determined by precipitation, washing in ethanol and weighing the precipitate. This figure is 278/micrograms/ml. Hence, 51.5 mg. dry mucus solids bind 1 microgram of glucose. Phrased differently, a 45-gram organism would have to secrete and recover about one-half its weight as dry mucus solids each hour to account for the rate of uptake observed at this concentration of glucose. It should be pointed out that the measurement of glucose retained on the filter is probably high, since less self-absorption of the sample would be expected. The comparable figure for binding in the case of glycine and mucus is 15.1 mg. mucus solids for each microgram of glycine.

Some check on the technique is provided by using Ca^{45} and measuring its retention. Kwart and Shashoua (1957) published a discussion of mucus structure in *Busycon,* giving the calcium content of mucus. Their proposed structure implies that this should exchange so that at least the equivalent of the calcium linking the protein and polysaccharide should be bound. Their figure for calcium in mucus extracted in NaCl is 10.3 μg./mg. solids. Using the technique above, 1 mg. mucus solids binds 8.4 μg. calcium, which is in reasonable agreement.

TABLE IV

Effect of phlorizin on uptake of glucose and glycine

I. Glucose (0.5 mg./l.) plus phlorizin

Phlor. concentration	% uptake (4 hours)
0	69
	67
10^{-5} *M*	32
	33
10^{-4} *M*	27
	30
10^{-3} *M*	0
	0

II. Glycine (0.25 mg./l.) plus phlorizin

Phlor. concentration	% uptake (4 hours)
0	89
	91
10^{-3} *M*	87
	90

A further test of the hypothesis was undertaken by plugging the mouth of specimens of *Fungia* with paraffin. Low-melting-point paraffin was melted and poured into and around the mouths of four animals. Their uptake of glucose from a solution of 0.37 mg./liter is indicated on Figure 2 by dark triangles. The points are plotted in such a manner as to compensate for the difference in ambient concentration. The rate of uptake is unaffected.

The preceding experiments rule out mucus binding as a mechanism, and suggest some form of transport across the body wall. Table IV reports the effect of phlorizin on uptake of glucose. The failure to inhibit uptake of glycine serves as a control. Glucose uptake is not inhibited by 10^{-3} molar 2,4-dinitrophenol.

Specificity of the pathway of uptake was investigated in two ways. A number of sugars were tested as possible competitive inhibitors of the uptake of glucose. The data are presented in Table V. It is clear that loading the system with these

TABLE V

Glucose uptake in presence of other sugars. Wet weight is 25–30 grams, initial glucose concentration is 1.25 mg./l., and initial concentration of other sugars is 500 mg./l.

Sugar	Uptake (%/hr.)
glucose alone	9.5 11.4
glucose + sucrose	14.3 10.2
glucose + galactose	14.4 13.6
glucose + ribose	11.6 13.6
glucose + arabinose	15.2 15.9

sugars does not affect the uptake of glucose. Observations were also made to determine whether these same sugars could be removed from solution by *Fungia* over a 24-hour period. Carbohydrate was determined using the anthrone technique as outlined by Lewis and Rakestraw (1955). Initial concentrations were 50 mg./liter, and animals weighing 30 to 40 grams were used. All of the glucose in 200 ml. of solution was removed after 24 hours. No uptake of any of the other sugars occurred. Hence, the pathway of uptake is rather specific for glucose.

Finally, it was of interest to determine the relation between temperature and rate of transport. Observations were made at several temperatures, ranging from 20° C. to 35° C. The effect of temperature on rate of uptake was modest. The Q_{10} for the range 20°–30° was 1.22; that for the range 25°–35° was 1.32. Initial concentration of glucose in these observations was 0.37 mg./liter. Another set of observations was undertaken at 100 mg./liter to determine if the temperature relations of the system differed at higher ambient concentrations. Measurements were made at 18°, 25°, and 32° C. The Q_{10} for the lower range was 1.19, and was 1.36 for the upper range.

A number of observations were undertaken using the techniques described but employing other compounds and other experimental animals. Some observations using glycine have already been mentioned. Table VI lists the percentage uptake

TABLE VI

Uptake of other organic compounds at the concentrations listed. Weight is 20 to 40 grams.

Compound	Conc. (mg./l.)	% uptake (4 hr.)
Tyrosine	0.15	89 70
Lysine	0.53	77 75
Aspartic acid	0.35	86 80
Glycine	1.2	96 83
Lactate	0.1	48 54

for five small organic compounds [4] at the concentrations stipulated. In each case, specimens of *Fungia* weighing from 20 to 40 grams were placed in 200 ml. of solution. Attention may be directed to two points of interest. The isoelectric point of lysine lies on the base side of the pH of sea water. Hence, both cations and anions can be taken up. Racemic mixtures of optically active amino acids were employed. Neither the data presented nor more frequent monitoring of ambient concentrations provide evidence for differential handling of D and L forms, though this remains possible.

A concentration curve for glycine differed from that reported above for glucose primarily in indicating a higher capacity of the system. At 250 and 600 mg./liter, 18 to 23 milligrams of glycine were removed from solution in four hours by animals weighing 30 to 40 gm. Blocking the mouth with paraffin was without effect on the rate of glycine uptake.

Observations on the uptake of glycine by several colonial corals indicated significant rates of uptake in all cases. Corals used included *Acropora* sp., *Favia speciosa,* and *Dendrophyllia micranthus. Dendrophyllia* is of interest in that it contains no symbiotic algae. Scattered observations on other phyla of reef-dwelling invertebrates indicate that the capacity to deal with small organic molecules in dilute solution is not limited to corals or to the Cnidaria.

DISCUSSION

The data reported strongly support the conclusion that *Fungia* is capable of removing several small organic molecules of biological significance from very dilute solution. Minimum concentrations of glucose and amino acids were dictated by the analytical technique and the specific activity of the labelled compounds employed. At these minimum concentrations, there was no apparent decline in the rate of uptake for the compounds employed, though absolute rate was of course a function of concentration. Hence, there is no reason to think that such uptake of small organic molecules does not occur at the very low concentrations one would expect in natural waters.

Not merely is glucose removed from dilute solution but this process occurs at a rate which is significant when compared with the energy requirements of the animal. It has been pointed out in presentation of the data that an ambient concentration of approximately 15mg./liter of glucose would provide sufficient material to support the observed oxygen consumption of the animals.[5] Furthermore, the observations concerning uptake of amino acids and lactate indicate that there is at least a modest spectrum of small organic molecules which can be effectively manipulated by the organism.

The preceding remarks should be balanced by a quite explicit statement that the relation of the present observations to any postulated nutritive significance of naturally occurring organic material is indirect. It is a prerequisite condition for utilization of organic material in true solution that an organism possess an effective collecting mechanism for such material. The present work demonstrates the existence of such a mechanism for some selected compounds. However, failing an adequate qualitative analysis of dissolved organic material, one cannot argue directly for a nutritive significance of these observations.

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[4] The compounds used were: DL-lysine-1-C^{14}, DL-aspartic acid-4-C^{14}, DL-tyrosine-2-C^{14}, glycine-2-C^{14}, and Zn lactate-1-C^{14}.

[5] The ambient concentration which is calculated as sufficient to support the observed oxygen consumption is somewhat higher than that previously reported (Stephens, 1960a). This difference springs primarily from the difference in oxygen consumption exhibited by the animals. The measurements reported here were made in May at a temperature of approximately 26°, those reported previously in December at a temperature of approximately 24°. In both cases, the stipulated concentrations lie in the general range of values reported for total dissolved organic material present in sea water.

Membrane transport of glucose is suggested by the present observations. Inhibition of transport by phlorizin has been classically reported for vertebrate gut and kidney. The specificity of the transport for glucose is also suggestive. However, the fact that galactose is not manipulated indicates that the transport system differs in some respects from that reported for other preparations (Crane and Mandelstam, 1960).

Active transport, in the sense of transport against a concentration gradient, cannot be drawn as a conclusion although it seems quite possible. If the data presented in Table I are interpreted on the basis of the naive assumption that the radioactivity of an extract is present as glucose, considerable concentration has clearly occurred. It is also true that Hosoi (1938) reported sugar concentrations of approximately 0.5% of dry weight in *Fungia actiniformis*. However, Hosoi's sugar was not identified, although glycogen was demonstrated. Neither is there support for the assumption concerning the form in which radioactive material was extracted. Even granting this dubious assumption and incomplete data in the literature, what glucose may be present in the animal is not necessarily osmotically active. Hence the question remains unresolved.

The low Q_{10} observed for glucose intake by *Fungia* contrasts with higher temperature coefficients typically reported for membrane transport systems. At low concentrations, this is not surprising, since transport would presumably be limited by the rate at which diffusion and mixing by flagellar activity could supply material, rather than by the transport mechanism itself. The failure to obtain a higher coefficient at the concentration of 100 mg./liter seems anomalous, however. Possibly this concentration did not exceed the capacity of the system sufficiently to overcome diffusion limitations.

The data presented concerning the retention of material adsorbed on samples of mucus indicate clearly that adsorption on mucus can provide only a trivial amount of the observed uptake. It is possible that the procedure of washing the mucus retained on the filter might permit the exchange of some material. However, the fact that plugging the mouth of the organism had no impact on the observed rate of uptake provides convincing evidence that this is not a major pathway of uptake.

While direct conclusions from the present work concerning the possible nutritive role of dissolved organic matter in natural waters must be eschewed, the existence of an efficient pathway for uptake of small organic compounds serves to renew interest in this hypothesis.

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RESPONSES FROM A PROPRIOCEPTIVE ORGAN OF THE CRAB, SESARMA RETICULATUM, DURING THE MOLT CYCLE[1]

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It has long been recognized that the various physiological and morphological changes associated with molting may occupy a considerable portion of the life of arthropods. During periods of growth, so-called intermolt stages are scarcely separated from preparations for, and recovery from, ecdysis. In attempting to assess the role of sensory receptors in the overall biology of the animal, there is need of quantitative information on the effect of these changes associated with molting on sensory input.

In fact, however, no arthropod sense organs have been so investigated. If sensory input remains essentially unaltered by the morphological changes during molting, this fact is of value both in extending our understanding of the functioning of the central nervous system to all adult stages of these animals, and in posing the problem of how such constancy is achieved in spite of replication of the exoskeleton. On the contrary, if sensory input does change markedly, this fact must be incorporated into our assessment of the functioning of the central nervous system.

The need of quantitative assessment of the effects of molting is particularly evident for the proprioceptive organs which signal position or movement of the exoskeleton-supported appendages. Several investigators have described such organs in brachyurans, beginning with Burke (1954), who described the propodite-dactylopodite joint organ (PDO). He recorded PDO activity both *in situ* and from the isolated organ, and using macroelectrodes demonstrated proprioceptive and "vibration sense" responses. Wiersma and Boettiger (1959) reexamined the PDO, and found that the organ was not acting as a vibration receptor but contained sense cells responsive unidirectionally to both position and movement. The PDO contains scolopale cells and is thus a chordotonal organ (Whitear, 1960), since this term is now used for any structure containing such cells (Pringle, 1960), in which the axial filaments of two bipolar sense cells end. With the electron microscope Whitear (1960) showed that one of the axial filaments contains a ciliary segment and is different from the other axial filament; the scolopale cell was thus termed heterodynal. Whitear (personal communication) now believes this scolopale cell to be a single cell and not a complex of scolopale cell, tube cell, and sheath cell as she has previously reported.

Burke, Wiersma and Boettiger, and Whitear employed *Carcinus maenas* as an experimental animal. Wiersma (1959) subsequently described the PDO in *Maia, Homarus,* and *Palinurus*, and counted the numbers of fibers responding

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to movement and position during opening and closing. In carrying out the work reported here the identification and description of the PDO is extended to another brachyuran family, the Grapsidae. The experimental approach is to demonstrate and study the response of the PDO in an intermolt crab, and then to compare the intermolt condition and response to crabs which have been forced into different stages of the molt cycle by eyestalk ablation.

MATERIALS AND METHODS

A. *Molting*

The marsh crab, *Sesarma reticulatum* (Say), (family Grapsidae) was employed as the experimental animal. Crabs were dug from tidal mud banks in New Haven, Connecticut, during mid-winter. These crabs were placed in individual glass containers, just covered with sea water (salinity 28‰), acclimated for two days at 15° C., and then maintained at 25° C. in a constant-temperature room. Water was changed every three days; the crabs were not fed. Animals were manipulated into "forced" proecdysis by ablation of their eyestalks, which causes an abrupt release of molting hormone (see Passano, 1960; Passano and Jyssum, unpublished data).

Proecdysis stages were determined by measuring the regeneration (R) of a walking leg autotomized concurrently with the ablation of the second eyestalk (Bliss, 1956):

$$R = \frac{\text{length of regenerating limb}}{\text{carapace width}} \times 100$$

Such R values are precisely related to the stage of the crab in the premolt cycle (Bliss, 1960). Animals dying were rated as having entered proecdysis (D_1) if they possessed a mucilaginous membranous layer (Drach, 1939; Passano, 1960). Of 50 eyestalkless crabs, 25 reached proecdysis and 8 reached exuviation.

PDO response was measured from isolated legs obtained by forced autotomy from experimental animals of different R values, postecdysial crabs, and intermolt crabs. Figure 1 shows the point at which a leg was removed to test the PDO. Since autotomy does not seriously affect the molt sequence (Passano, 1960), the same crab could be used as a leg donor for two molt stages. No apparently regenerated limbs were utilized since the PDO in these may possess an abnormal response (Wiersma, 1959).

B. *Histology*

The PDO was stained vitally with rongalit methylene blue and examined in intermolt, premolt, and postecdysial crabs. The organ was exposed, held with forceps and gently pulled away from its attachment, first distally and then proximally. If the organ pulled epidermis with it for at least 1 mm., it was termed "loose"; if not, the organ was term "intact." The PDO in animals of different R values and in postecdysial animals was compared to the PDO in intermolt crabs.

C. *Electrophysiology*

All recordings were made from the PDO nerve fibers in the meropodite (see Figure 2). The propodite and carpodite were immobilized on the side of a Petri dish in a vertical position with modeling clay (F), the dactylopodite remaining above the clay and thereby moveable, and the meropodite remaining below the clay. The exoskeleton of the meropodite and the two apodemes and their attached muscles were removed, allowing the nerve bundle to float free in Pantin's (1934) crustacean Ringer's solution (R). The nerves were then teased apart with sharpened tungsten needles and lifted individually or in small bundles into the air on a platinum wire electrode (E). The reference electrode was a platinum wire in the solution. Stimulating the PDO by moving the dactylopodite revealed a responding nerve bundle, which in preliminary experiments had been visually traced from the meropodite back to the PDO and which with methylene blue staining at the site of the organ was shown to contain nerve fibers from all of the PDO receptors. Stimulating the PDO revealed no other responding nerve bundles. In some experiments fibers running with the PDO group, but not coming from the PDO, were found to fire in proecdysis crabs with R values above 10: these were separated from the PDO group. The preparation was isolated from vibration by mounting it on a heavy steel plate (V) resting on sawdust.

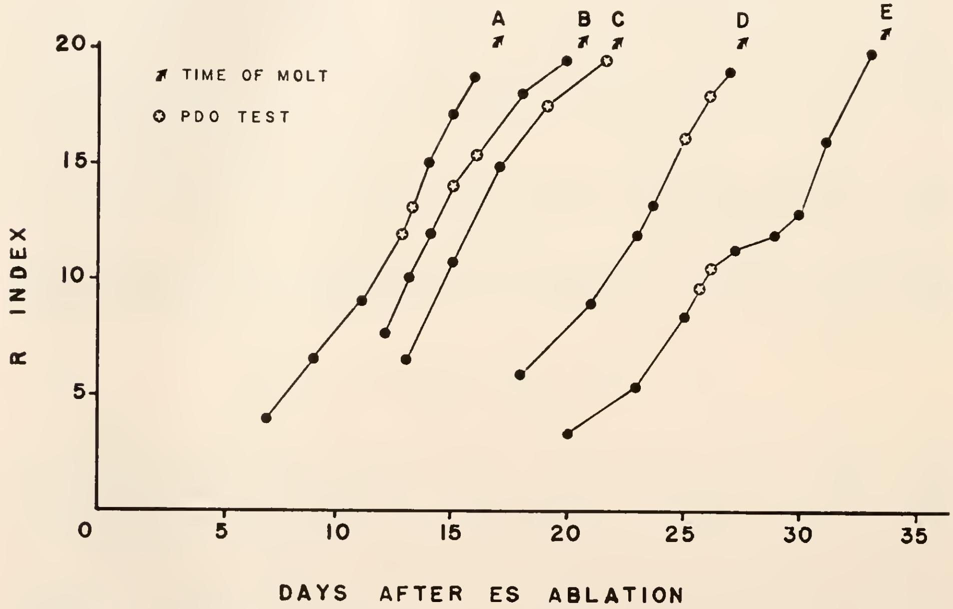

FIGURE 1. Proecdysis in eyestalkless (ES) *Sesarma,* as determined by limb regeneration (R; solid black circles). The starred circles represent the points at which a leg was removed to provide a PDO for testing; see Table I. Note that A-E represent different animals.

The signal was amplified (Tektronix 122 preamplifier; AMP), displayed on an oscilloscope, and recorded on tape. The tape (Scotch 109) moved at 3.75

inches per second on an Ampex 650 recorder. Output was also monitored audibly.

The dactylopodite was moved precisely and without vibration by the following method: A D.C. linear actuator motor (M) drove a steel shaft (S) back and forth. This shaft pulled a spring-loaded nylon thread (T) which entered a Faraday cage (C) and circumscribed a grooved aluminum wheel (W), which rotated on ball bearings on a fixed shaft. This wheel pushed a counterbalanced lever arm (L) whose end pivoted a 2-mm. diameter aluminum tube (Q) into which the dactylopodite (D) was fixed with dental cement. The counterbalanced arm provided a nearly weightless linkage. In order that the arm not displace the propodite-dactylopodite joint, the preparation was set so that the center of the joint was at the same height as the center of the wheel. The lever arm was then fixed by means of a sliding bolt (B), so that the radius r equaled r′, and the arc through which the dactylopodite was pushed was always the same and closely approximated its natural arc. With a two-way spring-loaded switch and a regulated power supply of adjustable output, the dactylopodite could be moved at any predetermined rate from 1.5°/sec. to 13.5°/sec. in either direction and held at any point. The total range of movement could be preset and automatically engaged. The nylon thread also turned a potentiometer (P) which was used to adjust the vertical position of the second beam on the oscilloscope, thereby recording the position of the dactylopodite. Since the motor and control apparatus were isolated from the preparation, and the moving parts were near frictionless, the movement of the dactylopodite was effectively vibration-free. For example a vibration-sensitive opening fiber which responded to a gentle stream of air on

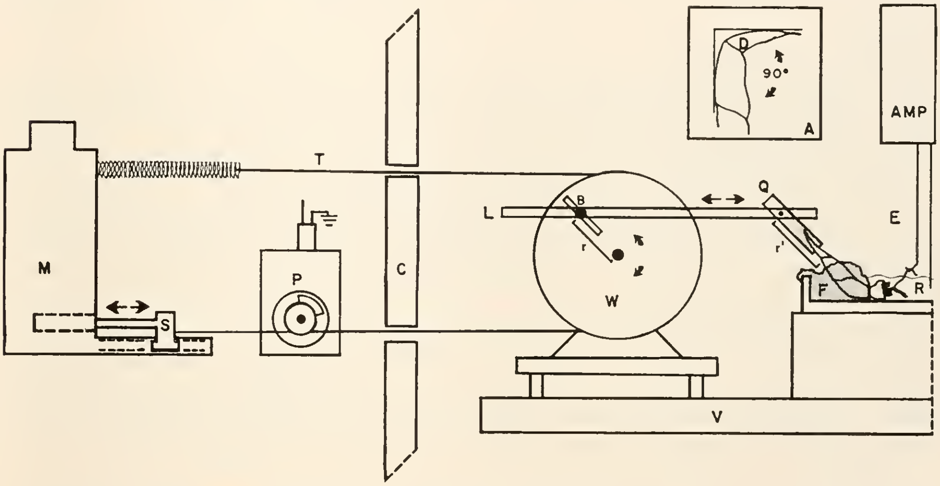

FIGURE 2. A diagram showing the mechanical stimulator and the recording situation: AMP, preamplifier; B, sliding bolt; C, Faraday cage; E, platinum electrode; F, clay; L, counterbalanced lever arm; M, motor; P, potentiometer; Q, tube cemented over dactylopodite; R, Ringer's solution; r and r′, equal radii; S, shaft; T, spring-loaded thread; V, vibration-free mounting plate. Insert 2A shows the dactylopodite (D) fully closed.

the dactylopodite did not respond while the dactylopodite was closed through 110°.

Preliminary experiments appeared to indicate that all PDO receptors, described in the results, were stimulated at a speed of movement of 8.58°/sec. through a range of 109.2°. This speed and range were used to obtain the results reported here. The fully closed position (where the dactylopodite was at 90° to the propodite, as indicated in insert, Fig. 2A) was taken as 0°. Fully open was thus 109.2° from the fully closed position.

The smoothness and evenness of the standard stimulus was shown by photographic analysis of the response of a movement fiber with a constant frequency of firing (Wiersma and Boettiger, 1959). However, photographic analysis allowed the determination of only a small number of the total PDO firings. Since isolation of single or small numbers of fibers leads to destruction of others, photographic analysis does not allow good quantitative analysis of total PDO response.

The method finally used was to play back a tape recording of the total opening and closing responses through a linear amplifier (TMC AL-2A) into a pulse counter (Berkeley 410) which counted the number of nerve spikes. Since the tape recorder amplifiers introduce some high frequency interference or "noise," the TMC amplifier gain was lowered to eliminate the noise. Thus, the final quantitative count is suitable for comparing the PDO from crabs of different R values, to intermolt and postmolt crabs, but the accuracy of the absolute number of nerve firings coming from the organ is limited by the discrimination of the counter.

In each experiment the dactylopodite was first closed fully. Starting with the resulting resting discharge, the experiment was taped while the dactylopodite was opened, held one second, and returned to the closed position; the motor switch artifact indicated when movement began and ceased. The gate-switch of the pulse counter was manually opened and closed when the tapes were played back for analysis.

The experimental recordings were played back into the pulse counter and analyzed in terms of the total number of firings accumulated over 109.2° of movement, both in opening and in closing. At least two runs were made with each preparation, but since there was an indication in some cases of slight diminution in total response with successive trials, the first runs were used for the quantitative comparisons. Individual runs were counted several times, but individual countings differed by less than 1% (± 30 counts).

RESULTS

A. *Histology*

A PDO corresponding in location and in dimensions to the PDO of *C. maenas* (Burke, 1954) was located in *S. reticulatum*. Vital staining of the organ revealed that in an intermolt crab with a 24.3-mm.-wide carapace, the PDO was 6.54 mm. long when the dactylopodite made a 90° angle with the propodite, and was 5.10 mm. long when the dactylopodite made a 125° angle with the propodite. The average diameter of the middle of the organ was 0.11 mm. There are about 40 cell bodies and their processes; 10 of these cell bodies are located more proximally and are larger than the other and more distal cell bodies. Unlike the

PDO of *Carcinus*, the PDO in *Sesarma* attaches distally without the presence of an inner protuberance from the exoskeleton of the dactylopodite. Rather, the organ seems to broaden and send off several strands of connective tissue to the adjacent epidermis within an area of 1 mm.^{2} from the point at which the main portion of the organ attaches. The PDO attaches proximally in the propodite by widening and meeting the epidermis at a point where the apodeme of the dactylopodite flexor muscle narrows sharply.

The condition of the distal and proximal attachments in crabs of different intermolt stages is summarized in Table I. Since the postmolt crab (A_1 stage) has such a soft exoskeleton, no accurate rating of "intact" or "loose" could be assigned to the organ's attachments during the first 5 hours after exuviation.

B. *General organ response*

The responses of individual units in the PDO of *S. reticulatum* are similar to the responses of units found in *C. maenas* by Wiersma and Boettiger (1959). The same physiological types were found: (a) large unidirectional phasic fibers responding with a constant frequency only to movement, (b) large unidirectional phasic fibers responding with an increase in frequency only to movement toward the fully open or closed positions, (c) large unidirectional phasic fibers responding only to movement at the extreme open or closed portions of the arc, (d) large adapting position fibers responding only at the fully open or closed positions, and (e) small tonic fibers—position fibers—responding at different positions in the arc. Unlike the situation reported by Wiersma and Boettiger in *Carcinus,* a position in which no fibers fire was not found. Occasionally opener and closer "c" category fibers were isolated as one bundle.

The above types of responses were found in crabs with R values from 10 through exuviation, in postmolt crabs, and in intermolt crabs. The responses thus do not vary qualitatively in those stages of the intermolt cycle that were examined.

C. *Total response of the PDO*

The total response and the state of attachment of the PDO in crabs with different R values, postmolt crabs, and intermolt crabs are presented in Table I. The source of the PDO from crabs of different R values is indicated in Figure 1.

As already noted above, the numbers of counts in Table I are from the first recorded run at each R value. Preliminary trials with intermolt animals showed an essentially constant response, within the experimental accuracy achieved here, between different legs of the same crab and also between legs of different crabs.

The only results which show any significant difference from the mean values obtained were those from the animal with the two lowest R values, *i.e.,* the earliest proecdysis stages measured. These values are significantly lower for responses to both opening and closing. The combination of probabilities gave a χ^2 of 20.6810, d.f 4, for an overall $P < 0.001$.

DISCUSSION AND CONCLUSIONS

The clear-cut conclusion from this study is that the intermolt PDO output does not vary markedly in crabs of R value 10 to exuviation, and in the immediate postexuvial stages. Since the analysis was limited to the first run of each experiment, the possibility of organ deterioration or damage to its attachments due to excessive stimulation was reduced.

It would help in understanding these findings if we knew how the PDO is attached to the integument. The transfer from the old to the new exoskeleton should be studied if the organ is attached to the exoskeleton beyond the epidermis. Burke (1954) in his drawings shows the distal end of the PDO attached to the exoskeleton above the epidermis in *C. maenas;* Whitear (personal communication) found that the epidermis in *Carcinus* can be teased away from the distal end, leaving the organ still connected to the integument. But decisions based on gross optical observations are liable to error. For example, the epidermis can be bound tightly to the membranous layer (particularly if *Carcinus* has reached anecdysis), and teasing the epidermis away from the organ does not prove the organ extends beyond the epidermis at its point of attachment. Greater resolution, as provided by the electron microscope, is needed; but no studies of this type have yet been published. Whitear (personal communication) has, however, examined the connections of a chordotonal organ crossing the meropodite-carpopodiate joining (MC_1) in *Carcinus*. Electron micrographs show this proprioceptive organ attaches onto the epidermis and does not extend through the "chitin-epidermis" junction, although the epidermis seems connected to the chitin by fibrous strands. That the PDO is so connected has not been demonstrated, but since a similar organ has been found to connect onto epi-

TABLE I

Comparison of PDO response and attachments during the molt cycle

Crab	R	Counts		Organ connection	
		Opener	Closer	Proximal	Distal
E	10.0	6128*	4098*	loose	intact
E	11.1	6092*	4045*	loose	intact
A	12.2	6420	4683	intact	intact
A	13.0	6743	4571	intact	intact
B	14.2	6923	4685	intact	intact
B	15.4	6395	4938	intact	intact
D	16.0	6728	4359	intact	intact
C	17.5	6865	4232	intact	intact
D	18.0	6791	4245	intact	intact
C	19.3	6651	4227	intact	intact
Postmolt	Hours				
	4	6587	4493	**	**
	5	6621	4719	**	**
	48	6699	4288	intact	intact
Intermolt (normal control)		6205	4320	intact	intact

* Significantly lower than combined other values.
** Animal too soft to determine.

dermis, but not beyond it, it is possible that the PDO is similarly attached solely to the epidermis.

If both ends of the PDO are connected only to the epidermis, then any change in response during proecdysis would be due to a difference in tension exerted on the organ during dactylopodite movement, or to changed mechanical properties within the organ. A loose peripheral connection would act as an elastic coupling in series with the stretched organ, and would take up some of the increased tension which must generate the PDO response. Either some mechanism allows the organ to respond similarly under different tensions, or the epidermis is never significantly separated from the remainder of the integument. Since dissections of the PDO of all stages showed an apparently normal organ without greatly altered connections, it is likely that the epidermis-exoskeleton separation does not occur in proecdysis stages corresponding to R values of 12 or more. It is only after excessive manual pulling that the organ and its attached epidermis pull away from the remainder of the integument. Since the response of the PDO does not vary markedly within the portion of the molt cycle investigated here, it seems probable that the tension in the organ which produces the response does not vary. The PDO would thus be independent of the state of the surrounding integument. This would be true whether the PDO attaches at the epidermis or extends beyond it, but is most easily understood if the organ is not attached beyond the epidermis.

Premolt growth is associated with crabs having R values greater than 10 (Bliss, 1960). Jyssum and Passano (1957) have shown two separate stages of limb regeneration: the first, basal limb growth, shows R values up to 10 and is molt-hormone-independent; the second stage, premolt growth, shows R values greater than 10 and is molt-hormone-dependent. But Bliss' results were from normal animals. Eyestalkless crabs molt in a shorter time than do normal crabs. Although the precipitous molt of eyestalkless *Sesarma* can still be halted when both Y-organs are removed at R value of 10 (Passano and Jyssum, unpublished data), it is clear that such animals are not precisely comparable to normal proecdysis crabs of the same R value in Drach stage D_0. An eyestalkless crab's R value 10 probably corresponds to stage D_1, for an eyestalkless crab's new dactylus is formed enough to be separated from its old exoskeleton at an R value of 12. Thus the range of data includes stages D_1 through B_1 and C_4 in Drach's terminology. It is during these stages that the PDO response has been measured and found to be constant.

Although not appreciated at the time these experiments were conducted, analysis of these data shows that the R values of the regenerating limbs of the experimental animals were lower than those from corresponding Drach stages of normal proecdysis crabs. This may have been due to the precipitous nature of eyestalk-removal-forced ecdysis, or of the failure to measure R values in regenerating limbs which had achieved basal limb growth before bilateral eyestalk extirpation, or from a combination of these two factors. Whatever the cause of this discrepancy, it resulted in a failure to test the PDO output in the earliest proecdysis stages, D_0 and early D_1. This is unfortunate, since there is a slightly smaller output from the earliest two stages tested, those in which the distal PDO attachment was termed "loose." But while significantly lower statistically, the reduction is less than 10% of the total PDO output; it is difficult to consider this reduction very important to the animal's total sensory input. These data were obtained from a single crab, and any conclusion should remain tentative. It is likely, however, that the loose distal connection found only at these stages allows some of the organ tension to be taken up by the displaced epidermis. The PDO response from crabs with R values lower than 10 should be examined to see if this slightly reduced response is truly characteristic of early premolt stages. But even if further work demonstrates that sensory input falls during the early stages of proecdysis, the fact remains that the sensory input from the PDO remains at a constant intermolt level during most of proecdysis, including the crucial period immediately around exuviation.

Although separate from the problem under investigation, one interesting finding should be noted. Several fibers firing with constant frequency were detected running with the PDO group in crabs of R value 10 or above; no such firings were noted in intermolt crabs. It is known that the tendon nerve (Wiersma and Boettiger, 1959) travels with the PDO bundle. Alexandrowicz (1957) has suggested that these tendon nerves, whose processes end somewhere in the integument, function to signal changes in the integument during molting. It is possible that these firings noted above originated in the tendon nerve cell bodies; this possibility should be explored.

I am indebted to Dr. L. M. Passano for his constant help and encouragement; I also wish to thank R. C. Morrison for his aid in quantitatively analyzing the data.

Summary

1. The identification of a proprioceptive organ (PDO), spanning the propodite-dactylopodite joint in the crab, *Sesarma reticulatum*, has extended the study of the PDO to the family Grapsidae.

2. The appearance and physiological response of the PDO in *Sesarma* are essentially identical to the appearance and response found by Wiersma and Boettiger (1959) in the *Carcinus* PDO.

3. A dactylopodite-moving stimulator is described; it provides a variable and controlled stimulus to the organ, allowing quantitative comparisons of the PDO output in proecdysis, postexuvial, and in intermolt stage crabs.

4. Sensory input from the PDO does not vary in proecdysis animals from Drach stage mid D_1 to exuviation, and in the immediate postexuvial stages, as compared to the response from intermolt crabs.

5. The constancy of the PDO output is discussed in terms of the organ's attachment to the integument.

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